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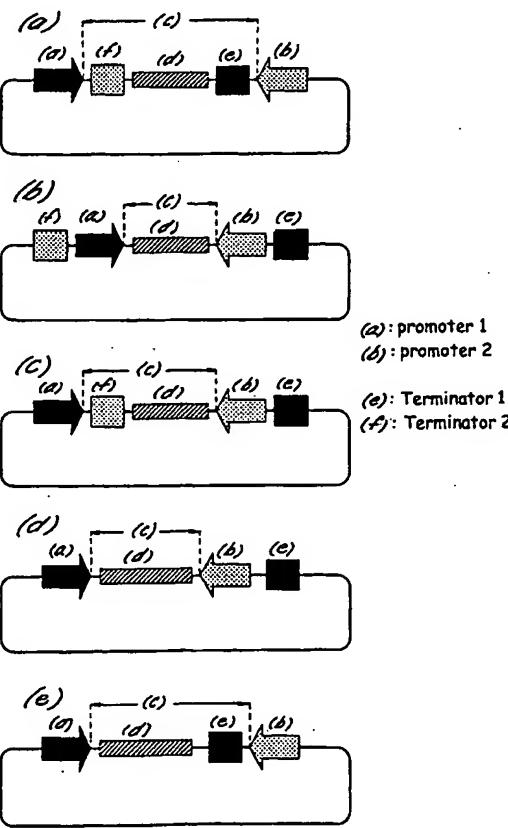
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(54) Title: VECTOR CONSTRUCTS



(57) Abstract: Vector constructs useful in the expression of double-stranded RNA. The constructs are particularly useful for expression of double-stranded RNA in vitro and in vivo.

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VECTOR CONSTRUCTSField of the invention

5 The invention relates to improved vector constructs for use in the expression of double-stranded RNA, particularly for use in the expression of double-stranded RNA *in vitro* and *in vivo*.

Background to the invention

10 Since the advent of double-stranded RNA inhibition (RNAi) as a tool for controlling gene expression, as described in WO 99/32619 and WO 00/01846, there has been recognised a need for specialised vectors designed for the production of 15 double-stranded RNA (dsRNA).

Cloning vectors designed to produce high levels of dsRNA have been previously described by Plaetinck et al. (WO 00/01846) and Timmons et al. *Nature*, 395:854 (1998). These vectors generally contain a 20 multiple cloning site (MCS) into which target DNA fragments can be cloned flanked by two opposable transcriptional promoters. Essentially, these three components (Promoter 1, MCS and Promoter 2) make up the entire system. In the appropriate expression 25 system, the DNA cloned into the MCS may be transcribed in both directions, leading to the production of two complementary RNA strands.

A disadvantage of the known systems is that not only the cloned fragment is transcribed. Read-through 30 of the RNA polymerase will result in transcription of the entire vector, and this also in both directions. As only transcription of the cloned DNA fragment will result in active dsRNA for RNAi purposes, transcription of the vector part results in useless,

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inefficient RNA. More specifically, 80% of these transcripts can be considered as non-specific and thus non-effective.

The large amounts of non-specific RNA generated by the prior art plasmid and expression systems results in some undesirable side effects. First, in RNAi protocols based on introduction of dsRNA into *C. elegans* via a food organism such as *E. coli* which expresses the dsRNA (see WO 00/01846), large RNA strands are considered to be toxic for the food organism. As a result, high amounts of RNA accumulating in *E. coli* cause a significant part of the population to die. Second, and probably more important, is the reduction of inhibition potential. The presence of large amounts of non-specific dsRNA causes a competitive environment for the specified sequences. The potential of the template-specified dsRNA sequences to inhibit the targeted protein expression in, for instance, *C. elegans* cells is reduced by the presence of these large non-specific regions. Such an inhibition by non-specific dsRNA has also been shown in *Drosophila* by Tushl et al., *Genes & Development* 13:3191-3197 (1999). Not only the potential to inhibit gene expression is affected, but also the amount of specific dsRNA produced is limited. Third, transcription of the vector backbone part, more particularly transcription of the origin of replication and related structures, results in plasmid instability and plasmid reorganisation, leading to reduced production of dsRNA. This relatively low concentration of effective dsRNA in turn leads to inefficient RNAi.

To conclude, the previously described vectors have following shortcomings: they are toxic to the

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feeding organism, a greater proportion of the transcripts produced are non-specific, the inhibitory potential of the dsRNA is reduced by the presence of non-specific regions, a high incidence of plasmid 5 reorganizations and loss of plasmid from the feeding organism. It is therefore an object of the present invention to provide improved vectors for the production of dsRNA which avoid the disadvantages of the prior art vectors.

10 Vectors for use in the *in vitro* synthesis of RNA transcripts, for example the production of RNA probes, have been known and commonly used in the art for some time (see for example F. M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994); Jendrisak et al, Vectors for *in vitro* production of RNA copies of either strand of a cloned DNA sequence, US 4,766072). In standard *in vitro* transcription protocols the problem of read-through transcription of vector sequences is generally 15 avoided by linearizing the transcription vector at restriction site positioned at the 3' end of the desired transcript. However, this solution is not appropriate for *in vivo* transcription or for the production of dsRNA where it is important that the 20 template is transcribed in both directions.

25 The inventors now propose a novel solution to the problems encountered with the prior art vectors for the production of dsRNA, based on the use of transcription terminators. Generally the solution 30 consists of the use of at least one transcription terminator operably linked to at least one promoter, wherein the terminator stops the transcription initiated by the promoter. Any DNA fragment inserted between the 3' end of the promoter and the 5' end of

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the terminator will then be transcribed, without the unwanted transcription of the vector backbone.

Preferentially the vector consists of two promoters and two terminators, as further described below.

5 Therefore, in accordance with a first aspect of the invention there is provided a DNA construct comprising two opposable promoters flanking an inter-promoter region, the construct further comprising at least one transcription terminator positioned
10 transcriptionally downstream of one of the said promoters. In particular, the invention provides for: a DNA construct comprising:

- a) a first promoter and
- b) a second promoter,

15 in which the first and second promoter are in opposite orientation to each other and define:

c) an inter-promoter region positioned downstream of the 3' end of the first promoter and downstream of the 3' end of the second promoter;

20 and which DNA construct further comprises:

d) at least one cloning site positioned in the inter-promoter region; and

e) a first transcription terminator, positioned (as seen from the 3' end of the first promoter)

25 downstream of the first promoter and downstream of the at least one cloning site, wherein the first transcription terminator is operably linked to the first promoter.

The inter-promoter region can also further be
30 defined as: the DNA region between the 3' end of the first promoter and the 3' end of the second promoter, and which is downstream of the first promoter, and which is downstream of the second promoter, and which preferably does not contain the 5' end of the first

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promoter and of the second promoter. The opposable first promoter and second promoter drive expression directional from their 5' ends to their 3' ends starting transcription downstream of their 3' ends, 5 thus providing transcription of both strands of any nucleotide sequence(s) present in the inter-promoter region.

The two promoters present in the DNA construct of the invention may be identical or they may be 10 different and may be of essentially any type. The precise nature of the promoters used in the construct may be dependent on the nature of the expression system in which the construct is expected to function (e.g. prokaryotic vs eukaryotic host cell). 15 Bacteriophage promoters, for example the T7, T3 and SP6 promoters, are preferred for use in the constructs of the invention, since they provide advantages of high level transcription which is dependent only on binding of the appropriate RNA polymerase. Each of 20 these promoters can independently be chosen. The phage promoters can also function in a wide variety of host systems, i.e. both prokaryotic and eukaryotic hosts, provided that the cognate polymerase is present in the host cell. 25 The arrangement of two "opposable" promoters flanking an inter-promoter region such that transcription initiation driven by one of the promoters results in transcription of the sense strand of the inter-promoter region and transcription 30 initiation driven by the other promoter results in transcription of the antisense strand of the inter-promoter region is an arrangement well known in the art, for example, in the pGEM7 series of vectors from Promega Corp., Madison WI, USA.

The DNA constructs of the invention differ from those of the prior art because of the presence of at least one transcription terminator positioned transcriptionally downstream of one of the promoters.

5 The transcription terminator may be uni- or bi-directional, the choice of uni- vs bi-directional terminators being influenced by the positioning of the terminator(s) within or outside the inter-promoter region, as explained below. The terminator may be of
10 prokaryotic, eukaryotic or phage origin.

Bacteriophage terminators, for example T7, T3 and SP6 terminators, are particularly preferred. The only requirement is that the terminator must be capable of causing termination of transcription initiating at the
15 promoter relative to which it is transcriptionally downstream. In practice, these means that the promoter and terminator must form a 'functional combination', i.e. the terminator must be functional for the type of RNA polymerase initiating at the
20 promoter. By way of example, a eukaryotic RNA pol II promoter and a eukaryotic RNA pol II terminator would generally form a functional combination. The selection of a functional combination is particularly important where bacteriophage promoters and
25 terminators are to be used in the constructs of the invention, since the phage promoters and terminators are both polymerase-specific. To form a functional combination both the promoter and the terminator should be specific for the same polymerase, e.g. T7
30 promoter and T7 terminator, T3 promoter and T3 terminator etc.

In one embodiment, the DNA construct of the invention may comprise a single transcription terminator, positioned (as seen from the 3' end of the

first promoter) downstream of the first promoter and downstream of the at least one cloning site, wherein the first transcription terminator is operably linked to the first promoter, wherein the single 5 transcription terminator is positioned in the inter-promoter region

In an alternative arrangement, the DNA construct comprises a single transcription terminator positioned outside of the inter-promoter region. In a still 10 further embodiment, the DNA construct may comprise two transcription terminators, each one of which is positioned transcriptionally downstream of one of the two promoters. In this arrangement, one or both of the terminators may be positioned within the inter-promoter region. These various embodiments of the DNA 15 constructs of the invention will be more fully described below, with reference to the accompanying drawings. The position of a first transcription terminator outside the inter-promoter region may also 20 be further defined as, i.e. such that a first transcription terminator is positioned (as seen from the 3' end of the first promoter) downstream of the first promoter, downstream of the at least one cloning site, and downstream of the 5' end of the second 25 promoter.

The position of a second transcription terminator outside the inter-promoter region may also be further defined as, i.e. such that a second transcription terminator positioned (as seen from the 3' end of the second promoter) downstream of the second promoter, downstream of the at least one cloning site, and downstream of the 5' end of the first promoter.

Moreover, when the terminator is not located in the inter-promoter region, the distance between the 5'

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end of the first promoter and 3' end of the second terminator, or the distance between the 5' end of the second promoter and the 3' end of the first terminator is preferably small, i.e. such that the 3' end of the 5 first transcription terminator is separated from the 5' end of the second promoter by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500 nucleotides, even more preferably no more than 200 nucleotides, 10 especially preferably no more than 100 nucleotides, more especially preferably no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides, particularly preferably no more than 10 nucleotides, more particularly preferably no 15 more than 6 nucleotides.

Furthermore, when the second transcription terminator is located outside of the inter-promoter region, preferably the 3' end of the second transcription terminator is separated from the 5' end 20 of the first promoter by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500 nucleotides, even more preferably no more than 200 nucleotides, especially preferably no more than 100 nucleotides, 25 more especially preferably no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides, particularly preferably no more than 10 nucleotides, more particularly preferably no more than 6 nucleotides

30 As defined above the term 'inter-promoter region' refers to all of the DNA sequence between the two promoters. As explained above, in certain embodiments of the invention the transcription terminator(s) may be sited within the inter-promoter region. The inter-

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promoter region may, advantageously, comprise a sequence of nucleotides forming a template for dsRNA production. The precise length and nature of this sequence is not material to the invention. The 5 invention further provides DNA constructs in which the inter-promoter region comprises a cloning site. The function of the cloning site is to facilitate insertion of a DNA fragment forming a template for dsRNA production between the two promoters. Thus, the 10 invention provides a series of cloning vectors which are of general use in the construction of template vectors for dsRNA production. Also encompassed within the scope of the invention are vectors derived from the cloning vectors which have a DNA fragment inserted 15 into the cloning site.

The cloning site may further comprise one or more of the following:

- at least one restriction site, (as known in the art), or one or more further restriction sites, 20 e.g. to provide a multiple cloning site (as known in the art),
- a stuffer DNA, e.g., flanked by at least two restriction site, such as two *Bst*XI restriction sites, or two *Xcm*I restriction sites,
- 25 - *attR1* and *attR2* recombination sites,
- a *ccdB* nucleotide sequence,
- a *ccdB* nucleotide further comprising at least one unique blunt-end restriction site, such as a *Srf*I restriction site, and/or
- 30 - a DNA fragment inserted in the at least one cloning site. All of the DNA constructs provided by the invention may, advantageously, form part of a replicable cloning vector, such as, for example, a plasmid vector. In addition to the opposable

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promoters, inter-promoter region and transcription terminator(s), the vector 'backbone' may further contain one or more of the general features commonly found in replicable vectors, for example an origin of 5 replication to allow autonomous replication within a host cell and a selective marker, such as an antibiotic resistance gene. The selective marker gene (e.g. the antibiotic resistance gene) may itself contain a promoter and a transcription terminator and 10 it is to be understood that these are completely independent of the promoter and terminator elements required by the invention and are not to be taken into consideration in determining whether a particular vector falls within the scope of the invention.

15 DNA constructs according to the invention may be easily be constructed from the component sequence elements using standard recombinant techniques well known in the art and described, for example, in F. M. Ausubel et al. (eds.), *Current Protocols in Molecular 20 Biology*, John Wiley & Sons, Inc. (1994), as will be appreciated by one skilled in the art from the following detailed description of the invention and the accompanying Examples.

25 There follows a detailed description of DNA constructs according to the invention, with reference to the following schematic drawings in which:

30 Figures 1(a) to 1(e) are schematic representations of several different embodiments of the DNA construct according to the invention illustrating the relative positioning of the promoter and transcription terminator elements.

Figure 2(a) is a schematic representation of a prior

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art vector included for comparison purposes.

Figures 2(b) to 2(e) are schematic representations of several further embodiments of the DNA construct
5 according to the invention illustrating the use of different cloning sites in the inter-promoter region.

Referring to the Drawings, Figure 1(a) schematically illustrates a first DNA construct
10 according to the invention which is a plasmid vector comprising two opposable promoters; a first promoter a) and second promoter b) flanking an inter-promoter region c), which inter-promoter region is downstream of the 3' of the first promoter, and downstream of the 3' end of the second promoter. The first promoter and the second promoter may be identical or different. This embodiment comprises a first transcription terminators e) and a second transcription terminator f) both of which are
15 positioned within the inter-promoter region. In this embodiment, the first terminator and the second terminator are preferentially uni-directional terminators.

A DNA fragment may be inserted in the at least
25 one cloning site d). Such fragment is subject to transcription directed by the first promoter a) and the second promoter b) (i.e. transcription of both strands), resulting in the generation of two RNA fragments which may combine to double-stranded RNA of
30 the inserted DNA fragment (both *in vitro* and *in vivo*).

Any desired DNA sequence, such as a genomic DNA sequence, or a cDNA sequence or any other coding sequence, may be inserted in the at least one cloning site. Without being limited to any specific

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explanation, it is assumed that when a) and e) form a functional combination, RNA polymerase which initiates transcription at a) will transcribe the inter-promoter region including the at least cloning site and the DNA 5 fragment inserted in the at least cloning site and will be terminated when it reaches e). Similarly, RNA polymerase which initiates transcription at b) will transcribe the inter-promoter region including the at least one cloning site and the DNA fragment inserted 10 in the at least one cloning site and will terminate when it reaches f). The terminators cause the RNA polymerase to pause, stop transcription and fall off the template. This prevents the unlimited transcription of the vector backbone, and reduces the 15 unspecific transcription of non-essential DNA.

The inter-promoter region further comprises a sequence of nucleotides corresponding to a target for double-stranded RNA inhibition. This sequence is designated 'TF' for target fragment. It is this 20 sequence which, when transcribed into dsRNA, will be responsible for specific double-stranded RNA inhibition of a target gene. The target fragment may be formed from a fragment of genomic DNA or cDNA from the target gene. Its precise length and nucleotide 25 sequence are not material to the invention.

In the arrangement shown in Figure 1(a) the two terminators are positioned on either side of the TF within the inter-promoter region. Each of the terminators is positioned transcriptionally downstream 30 of one of the promoters, the first terminator e) is transcriptionally downstream of first promoter a) and the second terminator f) is transcriptionally downstream of the second promoter b). Assuming that a) and e) form a functional combination, as described

above, RNA polymerase which initiates transcription at a) will transcribe the inter-promoter region up to and including TF and will be terminated when it reaches e). Similarly, RNA polymerase which initiates 5 transcription at b) will transcribe the inter-promoter region up to and including TF on the opposite strand and will terminate when it reaches f). The terminators cause the RNA polymerase to pause, stop transcription and fall off the template. This 10 prevents the unlimited transcription of the vector backbone, and reduces the unspecific transcription of non-essential DNA.

The transcripts generated from this vector may, depending on the precise placement of the terminators 15 in the vector, be almost completely specific dsRNAs corresponding to the TF region. Through the direct placement of the terminator sequences at the downstream end of the TF region on both sides of the inserted DNA fragment, the amount of material 20 transcribed is completely reduced to the template-specified sequences. Therefore, a higher amount of specific dsRNA is obtained. Furthermore the constructs are now also more stable, due to the non-transcription of the vector backbone. The latter 25 characteristic (stability), combined with the now relatively higher specific transcription rate, provides a system adapted to synthesise higher amounts of specific short dsRNA strands. This proportionally higher amount of transcript, resulting in high 30 concentrations of specific dsRNA, enhances the inhibitory effect in RNAi protocols. In RNAi protocols based on expression of dsRNA in a food organism, toxicity for the feeding organisms due to high RNA expression is brought to a minimal level by

use of this vector.

A specific example of a vector of the type illustrated in Figure 1(a), considered by the 5 inventors to be the optimal arrangement for RNAi applications, is plasmid pGN9 described in the accompanying Examples. The transcriptional terminators used in pGN9 are T7 RNA polymerase specific terminators, since the vector contains two 10 opposable T7 promoters. However, other systems could be used such as an expression system based on the T3 or SP6 promoter, terminator and polymerase or other prokaryotic or eukaryotic promoters and terminators.

Figure 1(b) illustrates schematically a further 15 DNA construct according to the invention which is a plasmid vector comprising two opposable promoters a) and b) flanking an inter-promoter region c). This vector also comprises two transcription terminators e) and f) but in this arrangement the two terminators are 20 positioned outside of the inter-promoter region, in fact the terminator elements now flank the two promoters. The arrangement is such that e) is transcriptionally downstream of a) whilst f) is transcriptionally downstream of b). Here again e) 25 terminates the transcription initiated by a), whilst f) terminates the transcription initiated by promoter b). Placement of the terminators outside of d) allows the use of bi-directional terminators as well as uni-directional terminators, in contrast to the 30 arrangement in Figure 1(a) where uni-directional terminators are preferred because of the placement of the terminators between the promoters. A number of bi-directional terminators which could be used in accordance with the invention are known in the art.

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Generally these are observed to be polymerase non-specific.

The embodiment shown in Figure 1(b) provides essentially the same advantages as that shown in 5 Figure 1(a) over the prior art vectors for dsRNA production. The vector shown in Figure 1(b) will lead to the production of transcripts which are slightly longer, including the promoter regions. This relatively small difference in the length of the 10 transcript and hence the formed dsRNA will not severely affect the efficacy in an RNAi system.

The position of the terminators and promoter in the example as shown in figure 1 (b) are preferably placed at close proximity, such that the 5' end of the 15 promoters are separated from the 3' end of the transcription terminators by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500 nucleotides, even more preferably no more than 200 nucleotides, 20 especially preferably no more than 100 nucleotides, more especially preferably no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides, particularly preferably no more than 10 nucleotides, more particularly preferably no 25 more than 6 nucleotides.

Figure 1(c) illustrates schematically a further DNA construct according to the invention which is a plasmid vector comprising two opposable promoters a) 30 and b) flanking an inter-promoter region c). In this embodiment one terminator (in this case e)) is positioned within the c) and the other (f)) is positioned outside c). Again, e) terminates transcription initiated by a) and f) terminates

transcription initiated by b). This arrangement may provide a useful solution to the problem of one of the terminators interfering with polymerase activity in the other direction (e.g. f) interferes with b) initiated transcription). This vector essentially provides the same advantages as the vector variations shown in Figure 1(a) and Figure 1(b) over the prior art. The relatively small difference in the length of the transcript due to the transcription of one of the promoters will not significantly affect the efficacy in RNAi systems. This more particularly the case when the terminator that is located outside of the inter-promoter region c) is at close proximity of the promoter, as defined above.

Figures 1(d) and 1(e) illustrate schematically two further DNA constructs according to the invention which are both plasmid vectors comprising two opposable promoters a) and b) flanking an inter-promoter region c). These embodiments comprise a single terminator only. In the arrangement shown in Figure 1(d) a single terminator e) which terminates transcription from a) is placed outside of c). The placement of the terminator outside of the IPR allows the use of both a bi-directional terminator or a uni-directional terminator in this system. In the embodiment shown in Figure 1(d) e) is placed within the c). a) should therefore preferably be a uni-directional terminator.

Further embodiments of the DNA construct according to the invention are illustrated schematically in Figures 2(b) to 2(e).

These embodiments are all plasmid cloning vectors, based upon the optimal arrangement of promoters and terminators shown in Figure 1(a), and

described above, containing cloning sites to facilitate the insertion of a DNA fragment into the at least one cloning site.

These embodiments are all plasmid cloning 5 vectors, based upon the optimal arrangement of promoters and terminators shown in Figure 1(a), containing cloning sites to facilitate the insertion of a target DNA fragment into the inter-promoter region.

10 Figure 2(a), which is a schematic representation of a prior art cloning vector, is included for comparison purposes. This vector comprises two opposable promoters a) and b), which may be identical or different, flanking a multi-cloning site (MCS).

15 Figure 2(b) illustrates a first type of plasmid cloning vector according to the invention. The vector contains a first opposable promoter a) and a second opposable promoter b) flanking an inter-promoter region. The inter-promoter region can further be 20 defined as: the DNA region between the 3' end of the first promoter and the 3' end of the second promoter, and which is downstream of the first promoter, and which is downstream of the second promoter, and which preferably does not contain the 5' end of the first 25 promoter and of the second promoter. The inter-promoter region further comprises terminators e) and f) flanking a multi-cloning site MCS. The MCS comprises at least one individual restriction sites, and preferably more than one 30 restriction site as known in the art, any of which may be used for insertion of a DNA fragment.

Figure 2(c) illustrates a further type of plasmid cloning vector according to the invention. This vector again contains opposable promoters a) and b)

flanking an inter-promoter region comprising terminators e) and f). In this embodiment, a) and b) flank a cloning site which is adapted for facilitated cloning of PCR fragments, comprising a stuffer DNA 5 flanked by two identical restriction sites, in this case BstXI sites. The specific sequence of the stuffer DNA is not essential, provided that said stuffer DNA does not interfere with the desired effect and/or the desired activity of the DNA constructs of the 10 invention. A specific example of a vector according to this aspect of the invention described herein is plasmid pGN29.

The cloning of PCR products using BstXI 15 recognition sites and BstXI adaptors is generally known in the art. The BstX1 adaptors are commercially obtained, such as from Invitrogen (Groningen, the Netherlands). These adaptors are non-palindromic adapters designed for easier and efficient cloning of PCR products into vectors. These use of these adaptors 20 reduces the concatemerization of adapters or insert DNA by having non-complementary (CACA) overhangs. The stuffer DNA is included merely to allow easy differentiation between BstXI cut and uncut vector on the basis of size. Its precise length and sequence 25 are not of importance.

Figure 2(d) illustrates a further type of plasmid cloning vector according to the invention. This vector again contains opposable promoters a) and b) flanking an inter-promoter region comprising 30 terminators e) and f). In this embodiment, a) and b) flank a cloning site which facilitates "High Throughput" cloning based on homologous recombination rather than restriction enzyme digestion and ligation.

As shown schematically in Figure 2(d), the cloning

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site comprises attR1 and attR2 recombination sites from bacteriophage lambda flanking a gene which is lethal to *E. coli*, in this case the ccdB gene.

An alternative cloning method of DNA fragments 5 into this vector, (not shown in Figure 2 (d)), consists of a variant of this vector, wherein the ccdB DNA sequence further comprises at least one unique restriction site, preferably the at least unique restriction site is a blunt-end restriction site, such 10 as a SrfI restriction site. Insertion of a DNA fragment in the at least unique restriction, results in inactivation of the ccdB gene, and hence in inactivation of the lethal ccdB gene.

A further variant of a vector a shown in Figure 15 2(d) in which the attR1 and the attE2 are not present. Such a vector comprises an at least one cloning site, said at least one cloning site consisting of a ccdB sequence, said ccdB sequence further comprising at least one unique restriction site, preferably the at 20 least unique restriction site is a blunt-end restriction site, such as a SrfI restriction site. Insertion of a DNA fragment in the at least unique restriction, results in inactivation of the ccdB gene, and hence in inactivation of the lethal ccdB gene.

25 These cloning sites comprising the ccdB nucleotide sequence and/or the attR sites (R1 and/or R2) are derived from the Gateway™ cloning system commercially available from Life Technologies, Inc. The Gateway™ cloning system has been extensively 30 described by Hartley et al. in WO 96/40724 (PCT/US96/10082). A specific example of a vector according to this aspect of the invention described herein is pGN39.

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Figure 2(e) and 2(f) illustrate a still further type of plasmid cloning vector according to the invention. This vector again contains opposable promoters a) and b) flanking an inter-promoter region 5 c) comprising terminators e) and f). In the embodiment shown in Figure 2(e), e) and f) flank a cloning site which facilitates "high throughput" cloning of PCR products by TATM cloning. This cloning site comprises a stuffer DNA flanked by two identical 10 restriction sites for an enzyme which generates overhanging T nucleotides. In this case the restriction sites are XcmI sites, but other sites which are cleaved to generate overhanging T nucleotides could be used with equivalent effect. The 15 overhanging T nucleotides facilitate cloning of PCR products which have a overhanging A nucleotide. This principle is known as TATM cloning. The cut vector with overhanging T nucleotides can be "topomerized" to generate a cloning vector of the type shown 20 schematically in Figure 2(f), by linking the enzyme topoisomerase to the overhanging T nucleotides. The resulting vector also facilitates the cloning of PCR products by the principle known as TOPOTM cloning.

Both the TOPOTM and TATM cloning systems, although 25 not for the vectors described in this invention, are commercially available from Invitrogen. The TOPOTM cloning system has extensively been described by Shuman in WO 96/19497 (PCT/US95/16099). The TATM cloning system has extensively been described by 30 Hernstadt et al. in WO 92/06189 (PCT/US91/07147).

It will be readily appreciated by the skilled reader that whilst Figures 2(b)-2(f) illustrate the inclusion of different cloning sites into a vector of the type illustrated in Figure 1(a), these cloning

sites could be included into any of the DNA constructs of the invention, including those illustrated schematically in Figures 1(b) to 1(e)

5 Application of the DNA constructs of the invention in RNAi technology.

As aforementioned, a major application of the DNA constructs/vectors of the invention is in the production of double stranded RNA for use in RNAi 10 technology. In particular, the constructs are useful in *in vivo* RNAi protocols in the nematode worm *C. elegans*.

In *C. elegans*, RNAi has traditionally been performed by injection dsRNA into the worm. Fire et 15 al. describes these methods extensively in International Application No. WO 99/32619. In short, both strands of RNA are produced *in vitro* using commercially available *in vitro* transcription kits. Both strands of RNA are allowed to form dsRNA, after 20 which the dsRNA is injected into *C. elegans*.

The new vector system developed by the present inventors is a drastic improvement on this traditional method. First, the RNA can be produced in one step, for instance by using two identical promoters such as 25 in the vector pGN9. Second, and more important, due to the presence of terminators the transcripts, and hence the formed dsRNA, will be more specific as only the cloned target fragment will be transcribed. This will result in a more efficient RNAi.

30 A further method to perform RNAi experiments in *C. elegans* has been described by Plaetinck et al. in WO 00/01846. In this method *C. elegans* worms are fed with bacteria which produce dsRNA. The dsRNA passes the gut barrier of the worm and induces the same RNAi

as if the dsRNA has been injected. For these experiments, the preferred *E. coli* strain is HT115 (DE3), and the preferred *C. elegans* strain is nuc-1;gun-1. The improved vectors provided by the 5 invention also improve the efficiency of RNAi in this method, as shown in the example below, as only effective dsRNA is produced.

Another method to perform RNAi has also been described by Plaetinck et al. in WO 00/01846. In 10 short, this method is based on the production of dsRNA in the worm itself. This can be done by using worm promoters in the described vectors, or by using a transgenic worm expressing a polymerase specific for non-*C. elegans* promoters present in the vector, such 15 that this polymerase drives transcription of the dsRNA. The promoters will preferentially be selected from the known bacteriophage RNA promoters, such as T7 or T3 or SP6 RNA promoters, which provide the advantage of a high level of transcription dependent 20 only on the binding of the cognate polymerase.

Plasmid vector DNA can be introduced into the worm by several methods. First, the DNA can be introduced by the traditional injection method (Methods in Cell Biology, Vol 48, *C. elegans* Modern 25 Biological Analysis of an organism, ed. by Epstein and Shakes). Second, the DNA can be introduced by DNA feeding. As has been shown by Plaetinck et al. in WO 00/01846, plasmid DNA can be introduced into the worm by feeding the worm with an *E. coli* strain that 30 harbors the plasmid. Preferentially the *E. coli* strain is OP50, or MC1061 or HT115 (DE3) but any other strain would suit for this purpose. The *C. elegans* strain is preferentially a nuc-1 mutant strain or a nuc-1; gun-1 strain. The plasmid DNA from the *E. coli*

passes the gut barrier and is introduced into the nematode, resulting in the expression of dsRNA. As with the other RNAi methods described above, the use of the new vector system will enhance the RNAi by the 5 production of only specific dsRNA.

The invention will be further understood with reference to the following experimental Examples, together with the following additional Figures in 10 which:

Figure 3 is a representation (plasmid map) of pGN1.

15 Figure 4 is a representation (plasmid map) of pGN9.

Figure 5 illustrates the nucleotide sequence of a fragment of plasmid pGN1, annotated to show the positions of the opposable T7 promoters.

20 Figure 6 depicts the nucleotide sequence of the T7 transcription terminator.

Figure 7 illustrates the sequences of 25 oligonucleotides oGN27, oGN28, oGN29 and oGN30 used to insert T7 transcription terminators into pGN1. The positions of the T7 pol terminator sequences and of various restriction sites are marked.

30 Figure 8 illustrates the nucleotide sequence of a fragment of plasmid pGN9, annotated to show the positions of the opposable T7 promoters and the T7 transcription terminators.

- 24 -

Figure 9 (a) is a representation (plasmid map) of pGN29; (b) is a representation (plasmid map) of pGN39; (c) is a representation (plasmid map) of the plasmid TopoRNAi.

5

Figure 10 shows the complete nucleotide sequence of plasmid pGN9.

10 Figure 11 shows the complete nucleotide sequence of plasmid pGN29.

Figure 12 shows the complete nucleotide sequence of plasmid pGN39.

15 Figure 13 shows the complete nucleotide sequence of plasmid TopoRNAi.

Figure 14 shows the complete sequence of plasmid pGN49A.

20

Figure 15 shows the complete sequence of plasmid pGN59A.

25

Figure 16 is a representation (plasmid map) of pGN49A.

Figure 17 is a representation (plasmid map) of pGN59A.

30

Example 1-Vector construction.

5 The starting point for construction of the vectors exemplified herein was plasmid pGN1. This plasmid, described in the applicant's co-pending International Application No. WO 00/01846, contains two opposable T7 promoters flanking a multi-cloning site.

10 Vector construction was carried out according to standard molecular biology techniques known in the art and described, for example, in F. M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

15

1) Construction of pGN9

pGN1 was first digested with restriction enzymes EcoRI and KpnI. Oligonucleotides oGN27 and oGN28 (Figure 7) were annealed to generate a double stranded 20 fragment which was then ligated into the EcoRI/KpnI cut vector. The resulting plasmid was re-digested with XbaI and HindIII. Oligonucleotides oGN29 and oGN30 were annealed to generate a double-stranded 25 fragment which was then annealed into the XbaI/HindIII cut vector. The resulting vector was designated pGN9 (Figures 4 and 10).

2) Construction of further cloning vectors

pGN29 (Figure 9(a); Figure 11) was generated by 30 replacing the MCS in pGN9 with a stuffer DNA flanked by BstXI sites. BstXI adapters are commercially available from Invitrogen (Groningen, the Netherlands).

pGN39 (Figure 9 (b); Figure 12) generated by following steps; pGN29 was digested with BstXI. BstXI adapters (Invitrogen, Groningen, The Netherlands) were ligated 5 to Cassette A provided by the GATEWAYTM system (Life Technologies, Inc.). Cassette A contains attR1, CmR, CcdA, CcdB, attR2. The Cassette A with the adapters where then ligated into the digested pGN29, resulting 10 in pGN39A. pGN39A contains a unique SrfI site in the ccdB gene.

The TopoRNAi vector (figure 9 (c); figure 13) was generated in the following way; pGN29 was digested with BstXI. Using PCR with the primers oGN103 and 15 oGN104 and template pCDM8 (Invitrogen, Groningen, The Netherlands), a stuffer was generated which includes XcmI sites. Onto the PCR product, BstXI adapters were ligated, and the resulting ligation product was ligated in the BstXI digested pGN29 vector resulting 20 in the TopoRNAi vector.

oGN103: 5' TACCAAGGCTAGCATGGTTATCACTGATAAGTTGG 3'

oGN104: 5' TACCAAGGCTAGCATGGCCTGCCTGAAGGCTGC 3'

25 PGN49A was constructed to insert an additional unique non-blunt restriction site and to delete the CmR gene pGN39. Overlap PCR was used. A first PCR was performed with primers oGN126 and oGN127 and PGN39A as template. Using primers oGN128 and oGN129 and the same template a 30 second fragment was generated. Overlap PCR using the resulting fragments and primers oGN126P and oGN129P resulted in a final PCR product. To this final PCR

- 27 -

product, BstXI adapters were ligated, and the ligation product was ligated into pGN29 digested with BstXI. The resulting vector was designated pGN49A.

5 A control vector was generated to test the efficiency of the pGN49A cloning vector, such vector should contain the T7 promoters, but not the T7 terminators. For this, the XbaI insert of pGN49A was isolated and cloned in pGN1 digested with the same restriction 10 enzyme. The resulting vector was designated pGN59A.

oGN126 pGATCTGGATCCGGCTTACTAAAAGCCAGATAACAGTATGC
oGN127 GGAGACTTTATCGCTTAAGAGAGACGTGCACTGGCCAGGGGGATCACC
oGN128:
15 CCAGTGCACGTCTCTTAAGCGATAAAAGTCTCCCGTGAACTTACCCGGTGG
oGN129 pGCTGTGTATAAGGGAGCCTGACATTATATTCCCCAG

Example 2-To illustrate the usefulness of the improved vectors in RNA.

20 This experiment was designed to illustrate the improved efficiency of the improved vectors of this invention in double-stranded RNA inhibition, as compared to the vectors known from the prior art. A significant increase on the efficacy of the system 25 could be expected, as more effective dsRNA was produced and hence RNAi performed better. The experimental system for this proof of concept experiment was to measure *C. elegans* rescue at 25°C in nuc-1 / pha-1(e2123)ts *C. elegans* mutants by RNAi of 30 sup35 using dsRNA feeding of pGN-2 (-terminator) and pGN-12 (+ terminator), with PGN-1 (empty vector) as a control and dilutor. The pha-1 ts / sup-35 mutation has extensively been described by Schnabel in WO

99/49066.

The *nuc-1* mutation in *C. elegans* provides for a *C. elegans* strain exhibiting better uptake abilities, 5 such as the uptake of the dsRNA complexes than wild type *C. elegans*. This mutant is deleted in the major DNase enzymes, and inventors have proven in earlier co-pending applications that this *C. elegans* strain results in enhanced RNAi by feeding the nematode with 10 dsRNAs.

The *pha-1(e2123)ts* mutation provides a mutant *C. elegans* strain with a phenotype of survival at 15°C and lethality at 25°C. This lethality is suppressible 15 by the inhibition of *sup-35* expression. RNAi of *sup-35* should thus facilitate the rescue of *pha-1(e2123)ts* at 25°C. The vectors of the present invention, when expressing dsRNA from *sup-35*, should increase the 20 efficacy of *sup-35* RNAi in rescuing *pha-1(e2123)ts* mutants at 25°C, compared to vectors that do not contain the terminators.

Vector pGN1 was used as empty vector. Vector pGN2 (-terminator) is a vector harboring *sup-35* DNA and 25 expressing *sup-35* dsRNA when introduced in the appropriate host, the vector has no transcriptional terminators inserted. Vector pGN12 (+ terminator) is a vector as described above, containing the transcriptional terminators, and hence resulting in 30 improved dsRNA production when introduced into an appropriate host. Thus, this vector has two unidirectional transcriptional terminators, both placed inside of the inter-promoter region, and flanking the *sup-35* fragment. Use of the latter

- 29 -

vector was expected to increase the efficacy of the system, here meaning a better rescue (survival) of pha-1(e2123)ts mutants at 25°C.

5 **Experimental conditions**

12-well micro-titer plates were filled with approximately 2 ml of NGM agar per well. (1 liter of NGM agar: 15g Agar, 1g peptone, 3g NaCl, 1ml cholesterol solution (5 mg/ml in EtOH), with 10 sterile addition after autoclaving of 9.5 ml 0.1M CaCl₂, 9.5 ml 0.1 ml MgSO₄, 25 ml 1M KH₂PO₄/K₂HPO₄ buffer pH 6, Ampicillin (100 µg/1), 5ml 0,1M IPTG and 5 ml nystatin solution (dissolved 10 mg/ml in 1:1 EtOH:CH₃COONH₄ 7.5 M)

15 The dried plates were spotted with approximately 50 µl of an overnight culture of bacteria HT115 (DE3) (Fire A, Carnegie Institution, Baltimore, MD) transformed with the plasmids. Individual nematodes at the L4 20 growth stage were then placed in single wells at day 1. In each well 1 nematode (P1). At day two, the P1 nematodes were placed to a new well and left to 25 incubate for a day. The same procedure was repeated at day 3. All plates were further incubated at 25°C to allow offspring to be formed. Sup35 RNAi induced survival (rescue) was measured by counting the offspring.

Results

30 RNAi experiment in *C. elegans* nuc-1/pha-1(e2123)ts mutants by feeding with *E. coli* expressing sup-35 dsRNA.

- 30 -

Set up:

pGN1 as control

pGN2 (sup 35 - Term.)

pGN12 (sup 35 + Term.)

5

pGN2 + pGN1 dilutions 1/2, 1/4, 1/8, 1/16, 1/32

pGN12 + pGN1 dilutions 1/2, 1/4, 1/8, 1/16, 1/32

10 Conditions:

Incubation temperature 25°C

Readout:

Count offspring (adult hermaphrodites)

pGN1 (control)

Day 1	0	0	0	0
Day 2	0	0	0	0
Day 3	0	0	0	0

pGN2 (undiluted)

Day 1	12	4	48	32
Day 2	24	23	80	85
Day 3	5	0	9	16

pGN12 (undiluted)

Day 1	16	29	37	14
Day 2	27	22	57	2
Day 3	1	2	4	1

pGN 2+1, 1/2 dilution

Day 1	0	7	0	2
Day 2	9	10	0	3
Day 3	0	2	0	0

pGN 12+1, 1/2 dilution

Day 1	22	28	103	61
Day 2	36	45	53	40
Day 3	3	3	25	1

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pGN 2+1, 1/4 dilution

Day 1	28	23	0	0
Day 2	6	3	0	0
Day 3	0	0	0	0

pGN 12+1, 1/4 dilution

Day 1	*	6	36	5
Day 2		24	55	3
Day 3				

pGN 2+1, 1/8 dilution

Day 1	0	0	4	0
Day 2	0	0	11	0
Day 3	0	0	0	0

pGN 12+1, 1/8 dilution

Day 1	31	12	16	38
Day 2	4	5	37	4
Day 3	0	0	2	1

pGN 2+1, 1/16 dilution

Day 1	0	0	0	0
Day 2	0	0	0	1 little
Day 3	0	0	0	0

pGN 12+1, 1/16 dilution

Day 1	1	0	0	0
Day 2	2	0	0	1
Day 3	0	1	1	1

pGN 2+1, 1/32 dilution

Day 1	0	0	0	0
Day 2	0	0	0	0
Day 3	0	0	0	0

pGN 12+1, 1/32 dilution

Day 1	0	0	1	0
Day 2	0	L2	3	0
Day 3	2	0	L3- L4	0

Conclusions

As expected, worms fed by bacteria harboring pGN1, did not result in the viable offspring, due to the lethal effect of the pha-1 mutation at this temperature.

5 Feeding the nematodes with *E. coli* harboring pGN2 or pGN12 both result in viable offspring. This is due to the feeding of the worm with dsRNA from sup-35. The remarkable difference between the two feeding experiments can be seen in the dilution series. When 10 diluting the bacteria harboring pGN2 with bacteria harboring pGN1, the number of offspring diminishes drastically, even at a low dilution of one to two. This dilution series indicates that high levels of dsRNA are needed to have a proper RNAi induction. In 15 the feeding experiment with bacteria harboring pGN12, significant offspring is still observed at a dilution of one to eight. This indicates that in the bacteria harboring pGN12, much more effective dsRNA is formed. This experiment clearly shows that the addition of 20 terminator sequences in vectors to express dsRNA as described above provide a significant advantage in the generation of RNAi.

Example 3: Comparison of RNAi efficiency of vectors
25 with and without T7 terminators (pGN49 vs pGN59)

Three different genes have been cloned in the vectors pGN49A and pGN59A. The cloning was performed by amplifying the gene fragments with *PfuI* DNA polymerase 30 producing blunt ends, facilitating cloning in these vectors. These PCR fragments were cloned into the vectors digested with *SrfI*. Correct fragment insertion of the clones was checked by PCR. The fragments are chosen such that ds expression and RNAi results in a

lethal phenotype of the offspring. This procedure allows to compare fast and easy the efficiency of the two vectors pGN49 and pGN59 in RNAi.

plasmid	Gene (acedb)	Vector backbones
pGW5	B0511.8	pGN49A
pGW9	C01G8.7	pGN49A
pGW11	C47B2.3	pGN49A
pGW17	B0511.8	pGN59A
pGW21	C01G8.7	pGN59A
pGW23	C47B2.3	pGN59A

All the plasmids (pGW-series) are transformed in *E.coli* AB301-105 (DE3) bacteria by standard methodology. The bacteria are then grown in LB/amp at 37°C for 14-18h.

25 These cultures were centrifuged and the bacterial pellet dissolved in S-complete buffer containing 1mM IPTG and 100 µg/µl ampicilin.

30 In 96 well plates containing 100 µl S-complete (containing 1 mM IPTG and 100 µg/µl ampicilin final concentration) and 10 µl of bacteria solution, 3 nematodes were added at each well, the nematodes were at the L1 growth stage.

The plates were incubated at 25°C for 5-6 days. Each

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day the plates are inspected for development of the larvae and the production of F1 offspring.

5 Results

The RNAi was performed in eight-fold for each constructed plasmid. The results show that when T7 terminators are inserted into the vector backbone, the expected phenotype gives a 100% occurrence. When T7 terminators are not used the reproducibility can decrease up to 50%. As in the previous experiment, the results show that the addition of terminators significantly enhances RNAi performance.

DNA

fragment	B0511.8	B0511.8	C01G8.7	C01G8.7	C47B2.3	C47B2.3
Vector	pGN49A	pGN59A	PGN49A	pGN59A	pGN49A	pGN59A
Resulting						
plasmid	PGW5	PGW17	PGW9	PGW21	PGW11	PGW23
Percentage						
lethal	100	75	100	87.5	100	50
Percentage						
offspring	0	25	0	12.5	0	50

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PCR fragment generated by the primers oGN103 and
oGN104 on template pCDM8

TACCAAGGCT AGCATGGTTT ATCACTGATA AGTTGG
5 ATAAGTTGGT GGACATATTA TGTTTATCAG TGATAAAAGTG TCAAGCATGA
CAAAGTTGCA GCCGAATACA GTGATCCGTG CCGGCCCTGG ACTGTTGAAC
GAGGTCGGCG TAGACGGTCT GACGACACGC AAACTGGCGG AACGGTTGGG
GGTGCAGCAG CCGGCGCTTT ACTGGCACTT CAGGAACAAAG CGGGCGCTGC
TCGACGCACT GGCGAAGCC ATGCTGGCGG AGAACATACAC GCTTCGGTGC
10 CGAGAGCCGA CGACGACTGG CGCTCATTTC TGATCGGGAA TCCCGCAGCT
TCAGGGCAGGC CCATGCTAGC CTGGTACCA GCACAATGG

Overlap PCR Fragment, which was used to generate
15 pGN49A

gatctggatccggcttactaaaagccagataacagtatgcgtattgcgcgctg
attttgcgtataagaatataactgatatgtataccgaagtatgtcaaaaa
gaggtgtgctatgaagcagcgtattacagtacagttgacagcgcacagctatca
20 gttgctcaaggcatatatgatgtcaatctccggctggtaagcacaaccatg
cagaatgaagccgtcgctgcgtgccgaacgcgtggaaagcggaaaatcaggaa
gggatggctgaggtcgccggttattgaaatgaacggctctttgctgacgag
aacaggactggtaatgcagttaaaggttacacctataaaagagagagccg
ttatcgctgtttgtggatgtacagagtatattgacacgcggcgca
25 cggatggtgatccccctggccagtgcacgtctttaagcgataaagtctccc
gtgaactttaccgggtggtgcataatcgggatgaaagctggcgcatgtac
caccgatatggccagtgtgccgtctccgttatcgaaaagaagtggctgat
ctcagccaccgcgaaaatgacatcaaaaacgccattaacctgatgttctgg
gaatataaatgtcaggctcccttatacacagc

30

Claims:

1. A DNA construct comprising:
 - a) a first promoter and
 - 5 b) a second promoter,
in which the first and second promoter are in opposite orientation to each other and define:
 - c) an inter-promoter region positioned downstream of the 3' end of the first promoter and downstream of 10 the 3' end of the second promoter;
and which DNA construct further comprises:
 - d) at least one cloning site positioned in the inter-promoter region; and
 - e) a first transcription terminator, positioned (as 15 seen from the 3' end of the first promoter)
downstream of the first promoter and downstream of the at least one cloning site, wherein the first transcription terminator is operably linked to the first promoter.
- 20 2. A DNA construct according to claim 1, further comprising:
 - f) a second transcription terminator positioned (as seen from the 3' end of the second promoter)
25 downstream of the second promoter and downstream of the at least one cloning site.
wherein the second transcription terminator is operably linked to the second promoter.
- 30 3. A DNA construct according to claim 1 or 2, in which the first transcription terminator is positioned in the inter-promoter region.

4. A DNA construct according to claim 1 or 2, in which the first transcription terminator is positioned (as seen from the 3' end of the first promoter) downstream of the first promoter, downstream of the at least one cloning site, and downstream of the 5' end of the second promoter.
5. A DNA construct according to any one of claims 2, 10 3 or 4, in which the second transcription terminator is positioned in the inter-promoter region.
6. A DNA construct according to any of claims 2, 3 15 or 4 in which the second transcription terminator is positioned (as seen from the 3' end of the second promoter) downstream of the second promoter, downstream of the at least one cloning site, and downstream of the 5' end of the first promoter.
7. A DNA construct according to any one of claims 4, 25 5 or 6, in which the 3' end of the first transcription terminator is separated from the 5' end of the second promoter by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500 nucleotides, even more preferably no more than 200 nucleotides, especially preferably no more than 100 nucleotides, more especially preferably no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides, particularly preferably no more than 10

nucleotides, more particularly preferably no more than 6 nucleotides.

8. A DNA construct according to any one of claims 6
5 or 7, in which the 3' end of the second transcription terminator is separated from the 5' end of the first promoter by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500
10 nucleotides, even more preferably no more than 200 nucleotides, especially preferably no more than 100 nucleotides, more especially preferably no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides,
15 particularly preferably no more than 10 nucleotides, more particularly preferably no more than 6 nucleotides.
9. A construct according to any one of the preceding
20 claims wherein the first and the second promoter are identical.
10. A DNA construct according to any one of the claims 1 to 7 wherein the first and the second
25 promoter are non-identical.
11. A DNA construct according to claims 8 or 9 wherein the first promoter and the second
30 promoter are independently chosen from T7, T3 or SP6 promoters.
12. A construct according to any one of the preceding claims wherein the cloning site comprises at

least one restriction site.

13. A DNA according to claim 11 wherein the cloning site comprises at least two restriction sites
5 flanking a sequence of stuffer DNA.
14. A DNA construct according to claim 12 wherein the at least two restriction sites are identical.
- 10 15. A DNA construct according to any one of the claim 12 to 13 wherein the at least one restriction site or the at least two restriction sites restriction sites are *BstXI* sites.
- 15 16. A DNA construct according to any one of the claims 12 to 13 wherein the restriction sites are *XcmI* sites.
- 20 17. A DNA construct according to any one of the preceding claims wherein the cloning site further comprises *attR1* and *attR2* recombination sequences.
- 25 18. A DNA construct according to any one of the preceding claims wherein the cloning site further comprises a *ccdB* nucleotide sequence.
- 30 19. A DNA construct according to claim 17 wherein the *ccdB* nucleotide sequence further comprises at least one unique restriction site.
20. A DNA construct according to claim 18 wherein the at least one unique restriction site are blunt-end restriction sites.

21. A DNA construct according to claim 19 wherein the blunt-end restriction sites are *SrfI* sites.
- 5 22. A DNA according to any one of the preceding claims which further comprises:
 - g) a DNA fragment inserted in the at least one cloning site.
- 10 23. A DNA construct according to any one of the preceding claims which is a plasmid or vector.
24. A plasmid or vector as claimed in claim 23 having the nucleotide sequence illustrated in Figure 10,
15 Figure 11, Figure 12, Figure 13, Figure 14, or Figure 15.
25. Use of the DNA construct according to any one of the preceding claims for the production of
20 double-stranded RNA for RNA inhibition.
26. A bacterial strain harbouring the DNA construct according to any one of the preceding claims.
- 25 27. A bacterial strain according to claim 26, wherein
20 said bacteria strain is an *E. coli* strain.
28. Use of the bacterial strain according to claims
26 or 27 for the production of double-stranded
30 RNA for RNA inhibition.

FIG. 1(a)

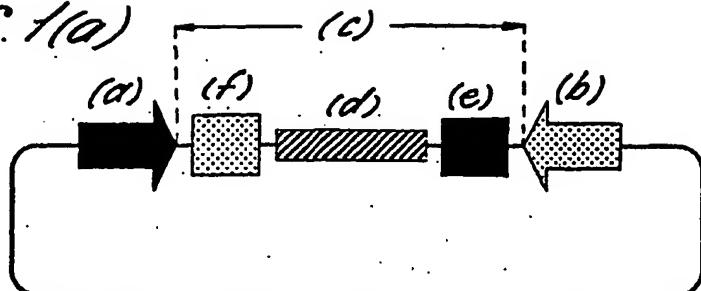
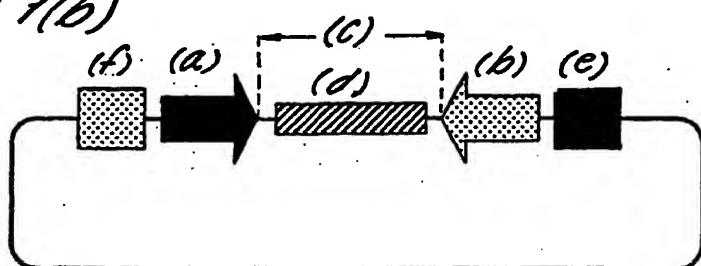


FIG. 1(b)



(a) : promoter 1
(b) : promoter 2

(e) : Terminator 1
(f) : Terminator 2

FIG. 1(c)

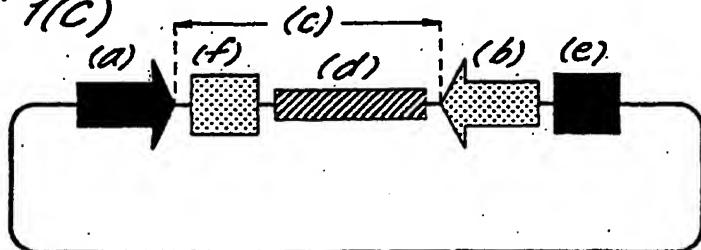


FIG. 1(d)

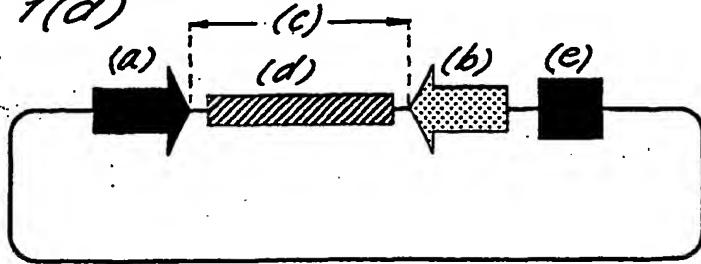


FIG. 1(e)

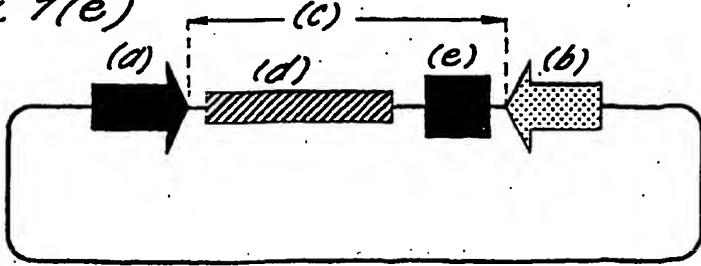


FIG. 2(a)

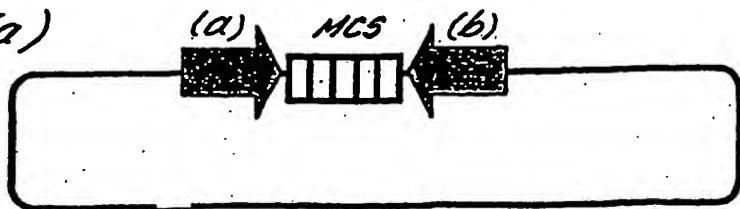


FIG. 2(b)

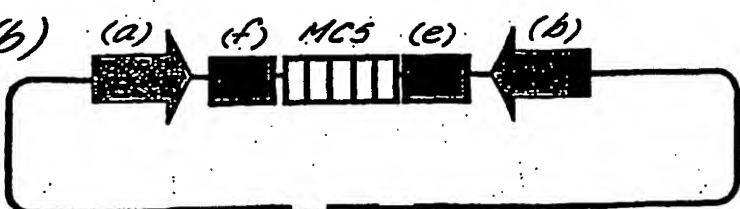


FIG. 2(c)

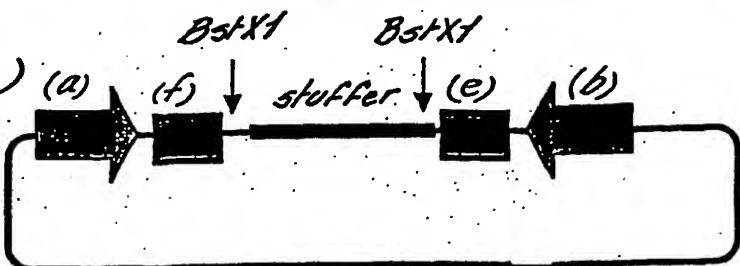


FIG. 2(d)

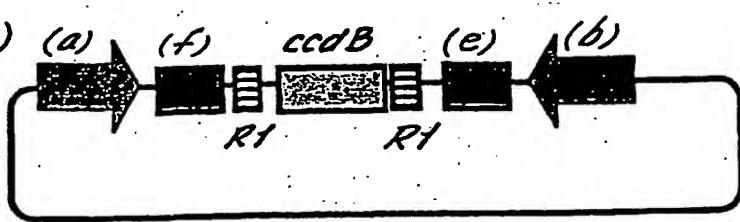


FIG. 2(e)

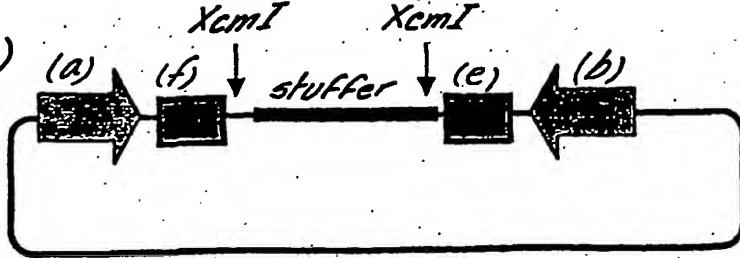
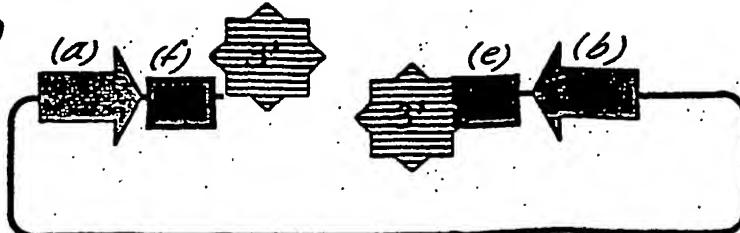


FIG. 2(f)



Construction RNAi vector with T7 terminators

FIG. 3.

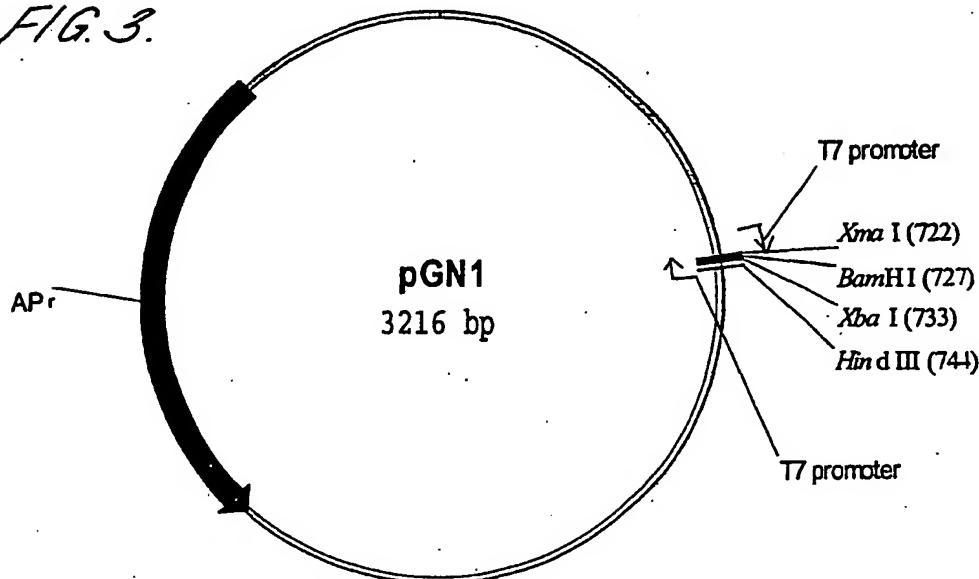


FIG. 4.

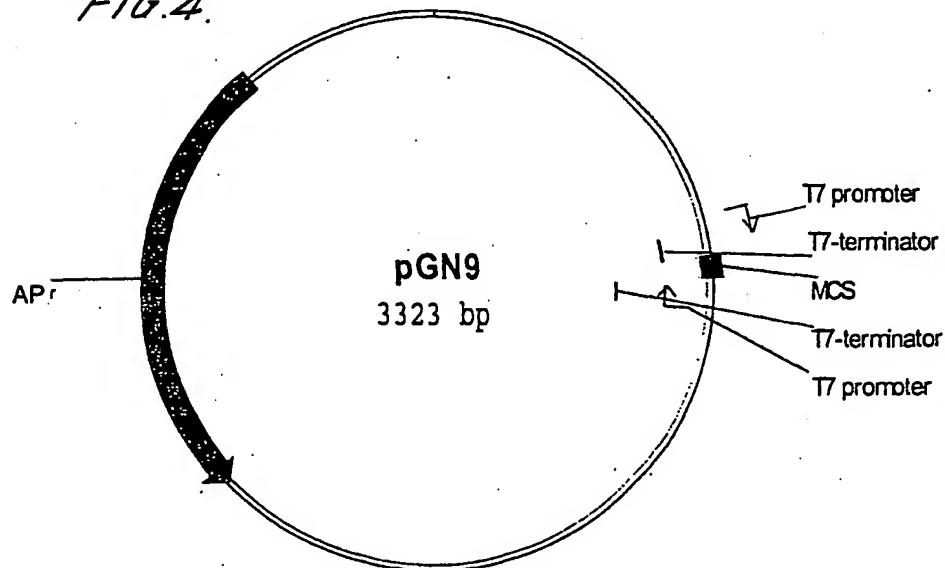


FIG. 5.

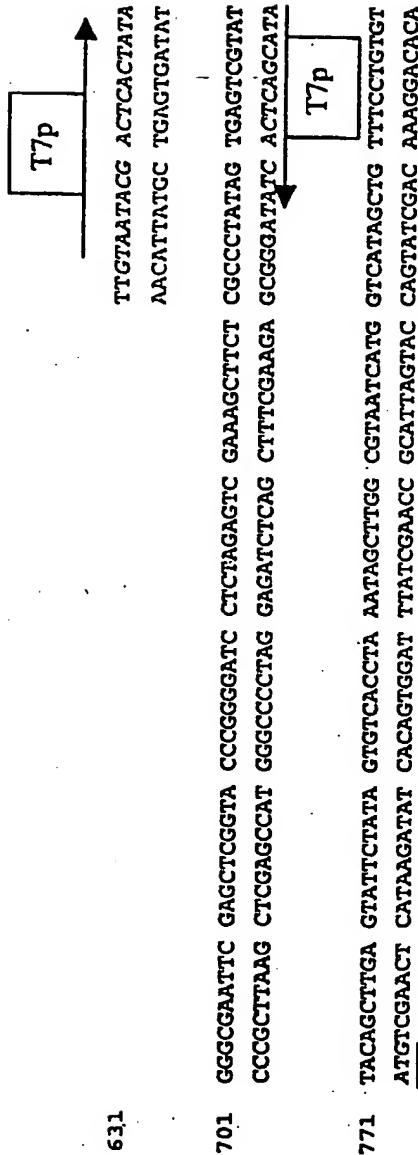


FIG. 6.

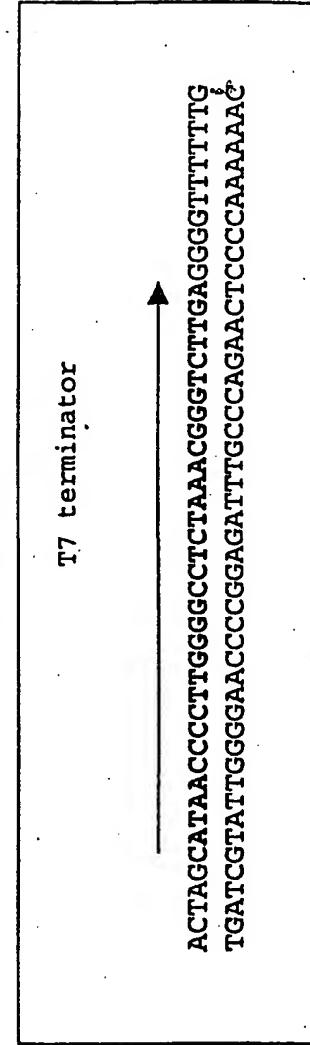


FIG. 7

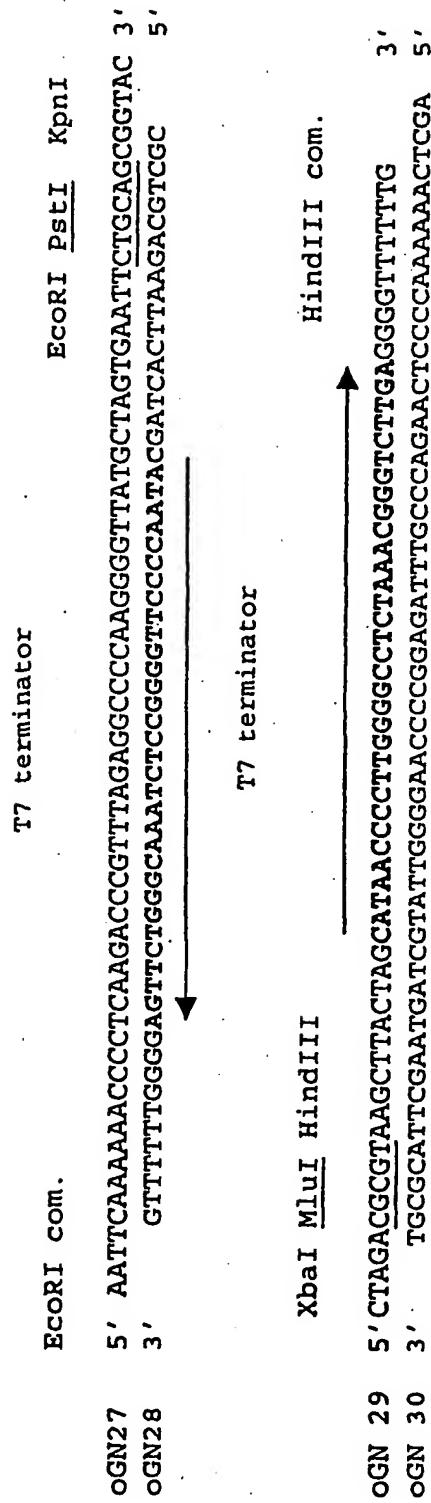


FIG. 8.

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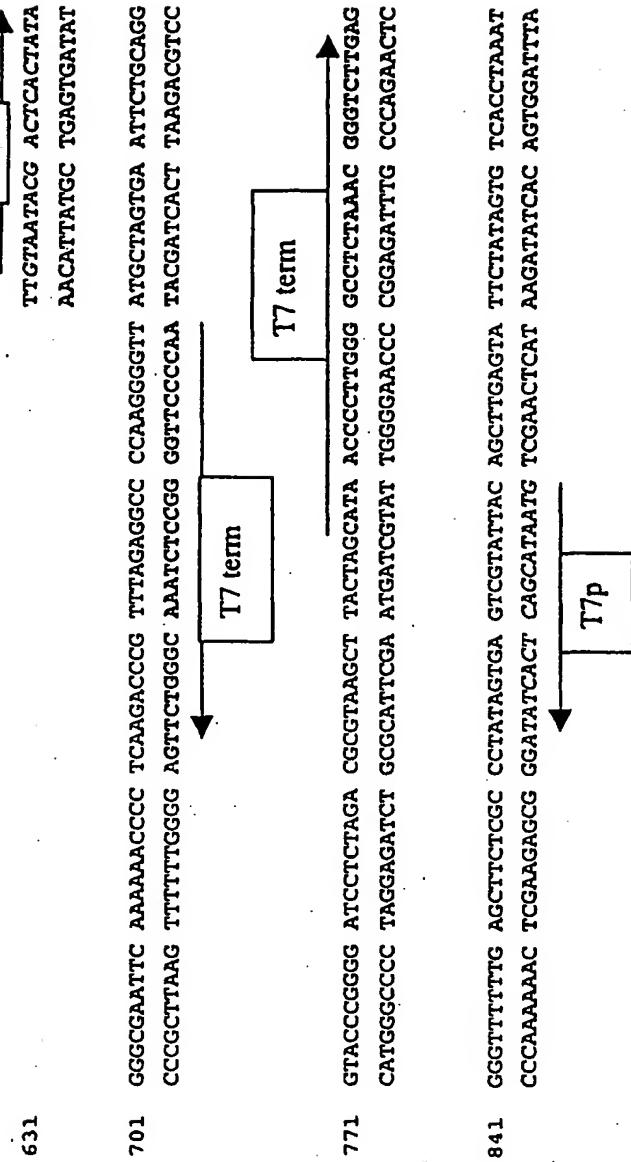


FIG. 9.

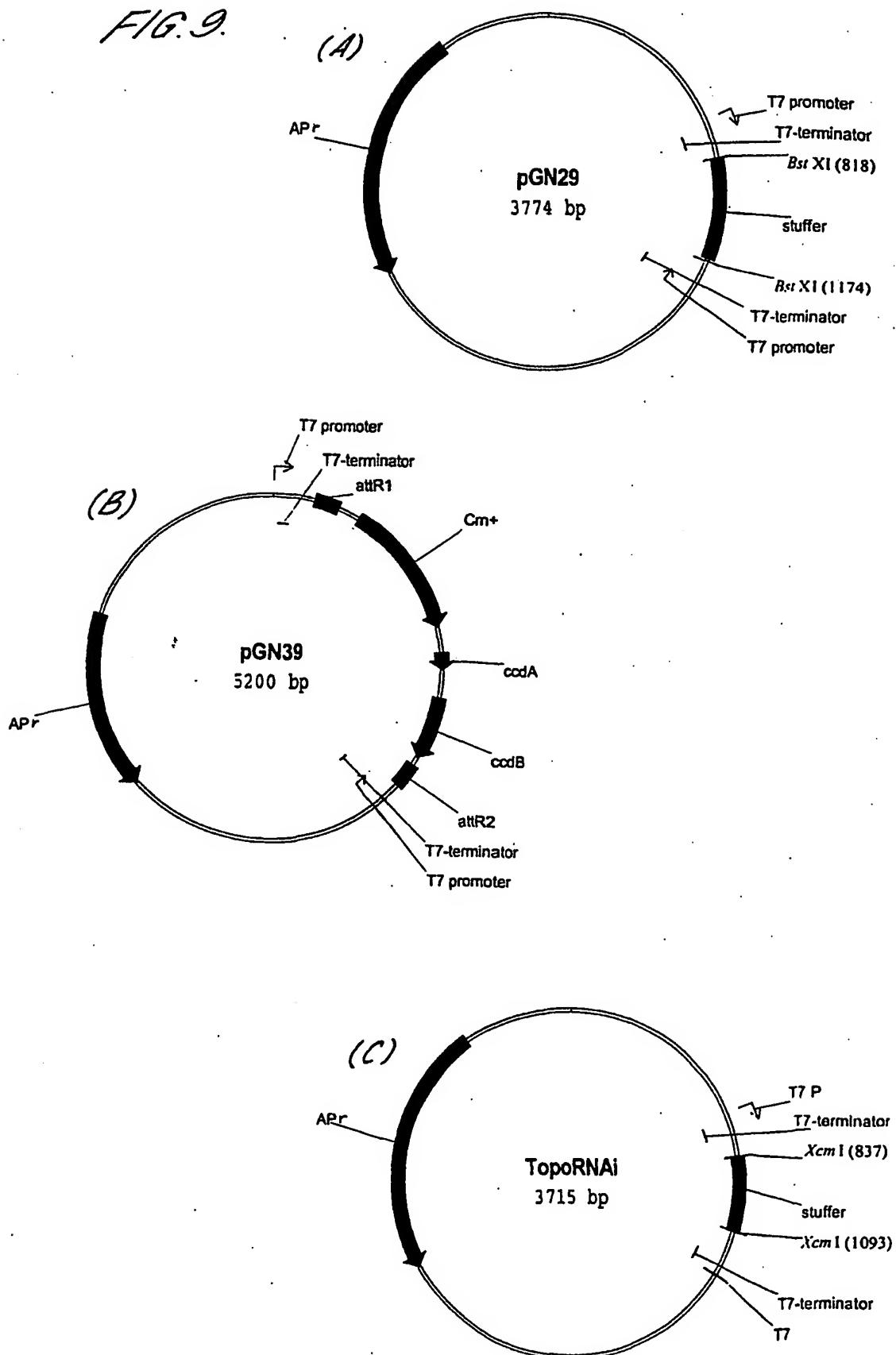


FIG. 10.

pGN9

1 gagtgcacca tatgcgggtgt gaaataccgc acagatgcgt aaggagaaaa taccgcatca
 61 ggcgaaattt taaacgttaa tattttgtta aaattcgcgt taaatattt ttaaatcagc
 121 tcattttta accaataggc cgaatccgc aaaatccctt ataaatcaa agaatagacc
 181 gagataggg ttagtgttgc tccagtttgc aacaagagtc cactattaaa gaacgtggac
 241 tccaaacgtca aaggcgaaa aaccgtctat cagggcgatg gcccactacg tgaaccatca
 301 cccaaatcaa gtttttgcg gtcgagggtgc cgtaaagctc taaaatcgaa ccctaaagg
 361 agcccccgat ttagagcttg acggggaaag ccggcgaacg tggcgagaaa ggaagggaag
 421 aaagcgaaaag gagcgggcgc tagggcgctg gcaagttgt tagtgcacgtc ggcgttaacc
 481 accacacccg ccgcgttaa tgcgcgccta cagggcgctg ccattcgcca ttcaaggctgc
 541 gcaactgtt ggaaggcgta tcggtgccgg ccttgcgtt attacgcccag ctggcgaaag
 601 ggggatgtc tgcaaggcga ttaagttggg taacgcagg gttttcccg tcacgacgtt
 661 gtaaaacgac ggccgtgaa ttgtatatacg actactata gggcgaattc aaaaaacccc
 721 tcaagacccg ttttagggcc ccaagggtt atgttagtga attctgcagg gtacccgggg
 781 atccctctaga cgcgtaaact tactagata accccttggg gcctctaaac ggtcttgag
 841 gggtttttgc agcttctcg cctatagtga gtcgttattac agtttagtga ttctatagtg
 901 tcacctaatt agcttggcgtaatcatggtc atagctgttt cctgtgtgaa attgttatcc
 961 gctcacaatt ccacacaaca tacgagccgg aagcataaaag tggtaaaggctt ggggtgccta
 1021 atgagtggc taactccat taattgcgtt ggcgtcaactg cccgccttcc agtccggaaa
 1081 cctgtcggtc cagctgttcaatgatccggc ccaacgcgcg gggagaggcg gtttgcgtat
 1141 tggcgcttcc tccgttccct cgtcaactg ctcgcgttgc tcggcggtt ggctcgccg
 1201 agccgtatca gctcaacttcc aaggcggtat acgggttatcc acagaaatcag gggataacgc
 1261 agggaaagaaac atgttagcaaa aaggccagca aaaggccagg aaccgtaaaaa aggccgcgtt
 1321 gctggcggtt ttgcgttaggc tccggccccc tgacgagcat cacaatccatc gacgctcaag
 1381 tcagagggttgg cgaaacccgta caggactata aagataccag gcttccccc ctggagc
 1441 cctcggtcgc tctccgttcc cgtaccgtcc gtttaccggta taccgttccg ctttctcc
 1501 ttcggaaacg gtggcgctt ctcatacgctc acgctgttgc tatctcgat ggtgttagt
 1561 cgttgcgttcc aagctgggtt gtgtcgacgaa ccccccgtt cagcccgacc gctgcgcctt
 1621 atcccgtaac tattgttccatc agtccaaaccg ggtttagtcc gacttacgc cactggc
 1681 agccactgtt aacaggatttta gcaagcgaaat gttatgttgc ggtgttccatc agttcttgg
 1741 gtgggtggcct aactacggct acactagaag gacagtattt ggtatctgcg ctctgtgaa
 1801 gccagttacc ttccggaaaaa gagttggtag ctcttgcattcc ggcaaaacaaa ccaccgc
 1861 tagcggtgtt tttttttttt gcaagcgacgca gattacgcgc agaaaaaaag gatctcaaga
 1921 agatcttgc tatttttca cggggctgtc cgttcgttgc aacggaaaact cacgtttaagg
 1981 gatttttgtt atgagattttaa aaaaaggat ttttccatc tagtgcatttccatc attaaaat
 2041 aagttttaaa tcaatctaaa gttatatagtt gtaaacttgg tctgacagt accaatgc
 2101 aatcgttag gcaatctatc cagcgatctg tctatttcgt tcatccatg ttgcctgact
 2161 ccccgctgtc tagataacta cgtatccgggaa gggcttacca tctggccccc gtgctcaat
 2221 gataccgcga gaccacgcgt caccggctcc agatttatca gcaataaaacc agccagccgg
 2281 aaggcccgag cgcagaatgt gttctgcacac tttatccgc tccatccatc tattaaat
 2341 ttgcggggaa gcttaggtttt gtagtgcgtt cgttgcgttcc agttatagt ttgcgc
 2401 tgctacagc atcggtgtt cagctgtcgtt gtttggatg gtttgcgttcc gctccgg
 2461 ccaacgtatca agggcgatgtt catgttccccc catgttgcggg aaaaaggcg tagtgc
 2521 cggtcctccg atcggtgtca gaagtttttgc ggttgcgttcc agttatcgttcc
 2581 agcactgcattt aattcttta ctgtcatgcgtt atccgttgcgttcc tttatccgc
 2641 gtactcaacc aagtcttgcgtt gagaataccg cggccggcga ccgagggttgc
 2701 gtcaataccg gataatagtgtt tagtgcatttttca cagaacttta aagtgc
 2761 acgttcttgc gggcgaaaac ttttgcgttcc ttttgcgttcc ttttgcgttcc
 2821 accctctgtt gcaaccatcgtt gatcttgcgttcc ttttgcgttcc ttttgcgttcc
 2881 agcaaaaaca ggaaggaaaat gtttttttttccatc gtttgcgttcc ttttgcgttcc
 2941 aatactcata ctcttccccc ttttgcgttcc ttttgcgttcc ttttgcgttcc
 3001 gagcggtatc atatttttttttgcgttcc ttttgcgttcc ttttgcgttcc
 3061 tcccccggaaa gtttttttttccatc gtttgcgttcc ttttgcgttcc ttttgcgttcc
 3121 aataggcgatc atcagggatc ctttttttttccatc gtttgcgttcc ttttgcgttcc
 3181 ctgacacatc gacgttccggg agacgggtac agtttgcgttcc ttttgcgttcc
 3241 acaaggccgtt caggccgttcc gacgttccgggatc gtttgcgttcc ttttgcgttcc
 3301 ggcattcgttcc gatatttttttccatc gtttgcgttcc ttttgcgttcc ttttgcgttcc

FIG. 11.

PGN29

1 gagtgcacca tatgcggtgt gaaataccgc acagatgcgt aaggagaaaa taccgcacca
 61 ggcgaaattt taaacgttaa tattttgtta aaattcgcgt taaaatattt ttaaatcagc
 121 tcattttta accaataaggc cggaaatccgc aaaaatccctt ataaatcaaa agaatagacc
 181 gagatagggt tgagtgttgc tccagtttgc aacaagagtc cactataaa gaacgtggac
 241 tccaaacgtca aaggccgaaa aaccgttat cagggcgatg gcccactacg tgaaccatca
 301 cccaaatcaa gtttttgc gtcgaggtgc cgtaaagctc taaaatcgaa ccctaaaggg
 361 agcccccgat ttagagcttgc acggggaaag cccgcgaacg tggcgagaaa ggaagggaaag
 421 aaagcgaaag gagcggcgc tagggcgtg gcaagtgttag cggtcacgtc ggcgtaaacc
 481 accacaccccg ccgcgtttaa tgccgcgtc aaggcgatg ccattcgcca ttcaaggctgc
 541 gcaactgttgc ggaaggcgat tcgggtgcggg cctcttcgtt attacgcacg ctggcgaaag
 601 ggggatgtgc tgcaaggcga ttaagtgggg taacgcacgg gttttcccgat tcacgcacgtt
 661 gtaaaacgcg gcccgtgaa ttgatatacg actcaactata gggcgaattc aaaaaacccc
 721 tcaagacccg ttagaggcc ccaagggtt atgctgttaga attctgcagg gtacccgggg
 781 atcctctaga gatccctcgat cctcgagatc cattgtgtgc gccgcggattc tttatcactg
 841 ataagggtt ggacatatta tggttatcag tgataaaatgt tcaagcatga caaagttca
 901 gccgaataca gtgatccgtg cccgccttgc actgttgaac gaggtcgccg tagacggct
 961 gacgacacgcg aaactggcg aacgggttggg ggtgcacgtc ccggcgttta actggcactt
 1021 caggaacaag cgggcgtcgc tcgacgcact ggcgcgaagcc atgtcgccgg agaatcatac
 1081 gcttcgggtgc cgagagccgat cgacgactgg cgctcgttgc tgatcgggaa tcccgcacgt
 1141 tcaggcaggc ggtgtcgcc tccgcggc acaatggatc tcgagggtat ttccatcac
 1201 accaggcttcg cccctcgagg tccgcggcgc gactctctag acgcgtaaagc ttactagcat
 1261 aacccttgg ggcctctaaa cgggtcttgc ggggtttttt gaggttctcg ccctatagtg
 1321 agtctgttta cagttgtatc attctataatgt gtcaccaaaa tagcttggcg taatcatgtt
 1381 catagcttttgc tcctgtgtgc aattgttataatcg cgctcacaat tccacacaac atacgaggcg
 1441 gaagcataaa gtgttaagcc tgggtgcctt aatgagtgat ctaactcaca ttaattcgct
 1501 tgccgtcact gcccgttcc cagtcgggaa acctgtcgat ccagctgc taaatgt
 1561 gccaacgcgcg ggggagggc gtttgcgtc ttggcgctc ttccgcttcc tcgctactg
 1621 actcgctgcg ctgggtcgat cggctcgcc gagggtatc agtcactca aaggcggtaa
 1681 tacggttatc cacagaatca gggataacg cagggaaagaa catgtgagca aaaggccacg
 1741 aaaaggcccg gaaccgtaaa aaggccgcgt tgctggcgat tttcgatagg ctccgcccc
 1801 ctgacgagca tcacaaaaat cgacgctcaat gtcagaggtg gccggaaacccg acaggactat
 1861 aaagataccat ggcgttccccc cctggaaatgc ccctcgatc ctctctcgatcc
 1921 cgcttaccggg atacctgtcc gctttctcc cttcgaaag ctggcgctt ttcatacg
 1981 cacgtctgtatc gtatctcgt tgggtgttgc tgggtgttgc tcaagctggc ggtgtcacc
 2041 aacccttccgt tcagccgcac cgctcgccat tatccgtaa ctatcgatctt gaggatcaacc
 2101 cggtaagaca cgacttatcg ccactggcag cagccactgg taacaggatt agcagagcga
 2161 ggtatgttagg cgggtctaca gagttcttgc agtgggtggc taactacggc tacactagaa
 2221 ggacagtatt tggtatctgc gctctgtgc gtcctgtatc cttcgaaaaa agatgttgc
 2281 gctcttgcgtt cggcaacaa accaccgcgtt gtagccgtgg ttttttgc tgcacgcacg
 2341 agattacgcg cagaaaaaaa ggtatctcaat aagatcttgc ttttttgc acgggtctg
 2401 acgtctgtatc gaaacggaaaatc acgttgcgtt ggttttttgc ttttttgc tcaaaaaagga
 2461 ttttgcgttgc gtttgcgttgc aattaaaaat ggttttttgc ttttttgc ttttttgc
 2521 agttaaacttgc gtctgacatc taccatgtc taatcgttgc ggcacccatc ttttttgc
 2581 gtctatccatc ttcatccata gttgcgttgc tccgcgttgc ttttttgc ttttttgc
 2641 agggcttacc atctggcccc agtgcgttgc ttttttgc ttttttgc ttttttgc
 2701 cagattttatc agcaataaac cagccagccg gaaaggccgc ggcacccatc ttttttgc
 2761 cttttatccgc ttttgcgttgc totattaaat gtttgcgttgc ttttttgc ttttttgc
 2821 cagttatagat ttttgcgttgc gtttgcgttgc ttttttgc ttttttgc ttttttgc
 2881 cttttatccgc ttttgcgttgc gtttgcgttgc ttttttgc ttttttgc ttttttgc
 2941 ccatgttgcgtt caaaaaaatc gtttgcgttgc ttttgcgttgc ttttttgc ttttttgc
 3001 tggccgcgtt gtttgcgttgc ttttgcgttgc ttttgcgttgc ttttttgc ttttttgc
 3061 catccgtaaatc atgcttttgc ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttttgc
 3121 ggcggccggc accgagttgc ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgc
 3181 gcaagaactt ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgc
 3241 ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgc
 3301 catccgttac ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgc
 3361 aaaagggtatc aaggccgaca cggaaatgtt gtttgcgttgc ttttgcgttgc ttttgc
 3421 attgtatccatc ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgc
 3481 aaaataaaaca aatagggtt cccgcgttgc ttttgcgttgc ttttgcgttgc ttttgc
 3541 aaaccattat tatcatgaca ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgc
 3601 tggccgttgc ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgc
 3661 cagttgtct gtaaggccgtt gtttgcgttgc ttttgcgttgc ttttgcgttgc ttttgc
 3721 ttggccgggtt ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgcgttgc ttttgc

*FIG. 12.**PGN39*

TAATACGACT CACTATAGGG CGAATTCAAA AAACCCCTCA AGACCCGTTT
AGAGGCCCA AGGGTTATG CTAGTGAATT CTGCAGCGGT ACCCGGGGAT
CCTCTAGAGA TCCCTCGACC TCGAGATCCA TTGTGCTGGA AAGATCACAA
GTTTGTACAA AAAAGCTGAA CGAGAAACGT AAAATGATAT AAATATCAAT
ATATTAAATT AGATTTGCA TAAAAAACAG ACTACATAAT ACTGTAAC
ACAACATATC CAGTCACTAT GGCGGCCGCA TTAGGCACCC CAGGCTTTAC
ACTTTATGCT TCCGGCTCGT ATAATGTGTG GATTTGAGT TAGGATCCGG
CGAGATTTTC AGGAGCTAAG GAAGCTAAA TGGAGAAAAA AATCACTGGA
TATACCACCG TTGATATATC CCAATGGCAT CGTAAAGAAC ATTTTGAGGC
ATTTCACTCA GTTGCCTAAT GTACCTATAA CCAGACCGTT CAGCTGGATA
TTACGGCCTT TTAAAGACC GTAAAGAAAA ATAAGCACAA GTTTTATCCG
GCCTTTATTC ACATTCTTGC CCGCCTGATG AATGCTCATC CGGAATTCCG
TATGGCAATG AAAGACGGTG AGCTGGTGAT ATGGGATAGT GTTCACCCCTT
GTTACACCGT TTTCATGAG CAAACTGAAA CGTTTCATC GCTCTGGAGT
GAATACCACG ACGATTTCCG GCAGTTCTA CACATATATT CGCAAGATGT
GGCGTGTAC GGTGAAAACC TGGCCTATT CCCTAAAGGG TTATTGAGA
ATATTTTCTT CGTCTCAGCC AATCCCTGGG TGAGTTTCAC CAGTTTGAT
TTAAACGTGG CCAATATGGA CAACTTCTC GCCCCCGTT TCACCATGGG
CAAATATTTAC ACGCAAGCGC ACAAGGTGCT GATGCCGCTG GCGATTCAAG
TTCATCATGC CGTCGTGAT GGCTTCCATG TCGGCAGAAAT GCTTAATGAA
TTACAACAGT ACTGCGATGA GTGGCAGGGC GGGGCGTAAA GATCTGGATC
CGGCTTACTA AAAGCCAGAT AACAGTATGC GTATTGCGC GCTGATTTTT
GCGGTATAAG AATATATACT GATATGTATA CCCGAAGTAT GTCAAAAAGA
GGTGTGCTAT GAAGCAGCGT ATTACAGTGA CAGTTGACAG CGACAGCTAT
CAGTTGCTCA AGGCATATAT GATGTCAATA TCTCCGGTCT GGTAAAGCACA
ACCATGCAGA ATGAAGCCCG TCGTCTGCGT GCCGAACGCT GGAAAGCGGA
AAATCAGGAA GGGATGGCTG AGGTCGCCCG GTTTATTGAA ATGAACGGCT
CTTTTGCTGA CGAGAACAGG GACTGGTGAA ATGCAGTTA AGGTTTACAC
CTATAAAAGA GAGAGCCGTT ATCGTCTGTT TGTGGATGTA CAGAGTGATA
TTATTGACAC GCCCGGGCGA CGGATGGTGA TCCCCCTGGC CAGTGCACGT
CTGCTGTCAG ATAAAGTCTC CCGTGAACCTT TACCCGGTGG TGCATATCGG
GGATGAAAGC TGGCGCATGA TGACCACCGA TATGGCCAGT GTGCCGGTCT
CCGTTATCGG GGAAGAAGTG GCTGATCTCA GCCACCGCGA AAATGACATC
AAAAACGCCA TTAACCTGAT GTTCTGGGA ATATAATGT CAGGCTCCCT
TATACACAGC CAGTCTGCAG GTCGACCATA GTGACTGGAT ATGTTGTGTT
TTACAGTATT ATGAGTCTG TTTTTATGC AAAATCTAAT TTAATATATT
GATATTATA TCATTTACG TTTCTGTTTC AGCTTCTTG TACAAAGTGG
TGATCTTCC AGCACAAATGG ATCTCGAGGG ATCTTCCATA CCTACCAGTT
CTGCGCTGCA AGTCGCGGC CGCGACTCTA GACCGCTAAG CTTACTAGCA
TAACCCCTTG GGGCTCTAA ACGGGTCTTG AGGGGTTTT TGAGCTTCTC
GCCCTATAGT GAGTCGTATT ACAGCTTGAG TATTCTATAG TGTACCTAA
ATAGCTTGGC GTAATCATGG TCATAGCTGT TTCTGCTGT AAATTGTTAT
CCGCTCACAA TTCCACACAA CATACTGAGCC GGAAGCATAA AGTGTAAAGC

FIG. 12 (CONTINUED 1)

CTGGGGTGCC TAATGAGTGA GCTAACTCAC ATTAATTGCG TTGCGCTCAC
TGCCCCGTTT CCAGTCGGGA AACCTGTCGT GCCAGCTGCA TTAATGAATC
GGCCAACGCG CGGGGAGAGG CGGTTGCGT ATTGGCGCT CTTCCGCTTC
CTCGCTCACT GACTCGCTGC GCTCGTGT CGGCTGCGG CGAGCGGTAT
CAGCTCACTC AAAGGCGGTAA ATACGGTTAT CCACAGAATC AGGGGATAAC
GCAGGAAAGA ACATGTGAGC AAAAGGCCAG CAAAAGGCCA GGAACCGTAA
AAAGGCCGCG TTGCTGGCGT TTTTCGATAG GCTCCGCCCC CCTGACGAGC
ATCACAAAAA TCGACGCTCA AGTCAGAGGT GGCGAAACCC GACAGGACTA
TAAAGATACC AGGCCTTTCC CCCTGGAAAGC TCCCTCGTGC GCTCTCCGT
TCCGACCCCTG CCGCTTACCG GATAACCTGTC CGCCCTTCTC CCTTCGGGAA
GCGTGGCGCT TTCTCATAGC TCACGCTGTA GGTATCTCAG TTCGGTGTAG
GTCGTTGCGT CCAAGCTGGG CTGTTGTCAC GAACCCCCCG TTCAGCCCCA
CCGCTGCGCC TTATCCGGTA ACTATCGTCT TGAGTCCAAC CCGGTAAGAC
ACGACTTATC GCCACTGGCA GCAGCCACTG GTAACAGGAT TAGCAGAGCG
AGGTATGTAG GCGGTGCTAC AGAGTTCTG AAGTGGTGGC CTAACTACGG
CTACACTAGA AGGACAGTAT TTGGTATCTG CGCTCTGCTG AAGCCAGTTA
CCTTCGGAAA AAGAGTTGGT AGCTCTTGT CGGCAAAACA AACCAACCGCT
GGTAGCGGTG GTTTTTTGT TTGCAAGCAG CAGATTACGC GCAGAAAAAA
AGGATCTCAA GAAGATCTT TGATCTTTTC TACGGGGTCT GACGCTCAGT
GGAACGAAAA CTCACGTTAA GGGATTGGG TCATGAGATT ATCAAAAAGG
ATCTTCACCT AGATCCTTTT AAATTAAAAA TGAAGTTTA AATCAATCTA
AAGTATATAT GAGTAAACTT GGTCTGACAG TTACCAATGC TTAATCAGTG
AGGCACCTAT CTCAGCGATC TGTCTATTTTC GTTCATCCAT AGTTGCCGTGA
CTCCCCGTCG TGTAGATAAC TACGATACGG GAGGGCTTAC CATCTGGCCC
CAGTGCCTGCA ATGATACCGC GAGACCCACG CTCACCGGCT CCAGATTTAT
CAGCAATAAA CCAGCCAGCC GGAAGGGCCG AGCGCAGAAG TGGTCCTGCA
ACTTTATCCG CCTCCATCCA GTCTATTAAT TGGTGGCGGG AAGCTAGAGT
AAGTAGTTCG CCAGTTAATA GTTGGCGCAA CGTTGTTGGC ATTGCTACAG
GCATCGTGGT GTCACGCTCG TCGTTGGTA TGGCTTCATT CAGCTCCGGT
TCCCAACGAT CAAGGCGAGT TACATGATCC CCCATGTTGT GCAAAAAGC
GGTTAGCTCC TTGGTCCCTC CGATCGTTGT CAGAAGTAAG TTGGCCCGAG
TGGTATCACT CATGGTTATG GCAGCACTGC ATAATTCTCT TACTGTCATG
CCATCCGTAA GATGCTTTTC TGTGACTGGT GAGTACTCAA CCAAGTCATT
CTGAGAATAC CGCGCCCGGC GACCGAGTTG CTCTGCCCG GCGTCATAAC
GGGATAATAG TGTATGACAT AGCAGAACTT TAAAAGTGCT CATCATTGGA
AAACGTTCTT CGGGGCAGAA ACTCTCAAGG ATCTTACCGC TGGTGGAGATC
CAGTTCGATG TAAACCCACTC GTGCACCCAA CTGATCTTCA GCATCTTTTA
CTTTCACCAAG CGTTTCTGGG TGAGCAAAAAA CAGGAAGGCA AAATGCCGA
AAAAAGGGAA TAAGGGCGAC ACGGAAATGT TGAATACTCA TACTCTTCCT
TTTCAATAT TATTGAAGCA TTTATCAGGG TTATTGTCCT ATGAGCGGAT
ACATATTTGA ATGTATTTAG AAAATAAAC AAATAGGGT TCCGCGCACA
TTTCCCGAA AAGTGGCACC TGACGTCATA GAAACCATTA TTATCATGAC
ATTAACCTAT AAAAATAGGC GTATCAGGAG GCCCTTTCGT CTCGCGCCGT
TCGGTGTGATGA CGGTGAAAC CTCTGACACA TGCAGCTCCC GGAGACGGTC
ACAGCTTGTG TGTAGCGGA TGCCGGGAGC AGACAAGCCC GTCAGGGCGC
GTCAGCGGGT GTGGCGGGT GTCGGGGCTG GCTTAACCTAT GCGGCATCAG

FIG. 12 (CONTINUED 2)

ACCGAGATTGT ACTGAGAGTG CACCATATGC GGTGTGAAAT ACCGCACAGA
TGCCTAAGGA GAAAATACCG CATCAGGCGA AATTGTAACG GTTAATATTT
TGTAAATT CGCGTTAAAT ATTTGTTAAA TCAGCTCATT TTTAACCAA
TAGGCCGAAA TCGGCAAAT CCCTTATAAA TCAAAAGAAT AGACCGAGAT
AGGGTTGAGT GTTGTCCAG TTTGGAACAA GAGTCCACTA TTAAAGAACG
TGGACTCCAA CGTCAAAGGG CGAAAAACCG TCTATCAGGG CGATGGCCCA
CTACGTGAAC CATCACCCAA ATCAAGTTTT TTGCGGTGCA GGTGCCGTAA
AGCTCTAAAT CGGAACCTA AAGGGAGCCC CCGATTTAGA GCTTGACGGG
GAAAGCCGGC GAACTGGCG AGAAAGGAAG GGAAGAAAGC GAAAGGAGCG
GGCGCTAGGG CGCTGGCAAG TGTAGCGTC ACGCTGCGCG TAACCAC
ACCCGCCGCG CTTAATGCGC CGCTACAGGG CGCGTCCATT CGCCATTCA
GCTGCGCAAC TGTGGGAAG GGCGATCGGT GCGGGCCTCT TCGCTATTAC
GCCAGCTGGC GAAAGGGGGA TGTGCTGCAA GGCGATTAAG TTGGGTAACG
CCAGGTTTT CCCAGTCACG ACGTTGTAAG ACGACGGCCA GTGAATTG

FIG. 13.

TopoRNAi

1 gagtgcacca tatgcggtgt gaaataccgc acagatgcgt aaggagaaaa taccgcacca
 61 ggcgaaattt taaaacgttaa tattttgtta aaattcgcgt taaaatatttgc taaaatcagc
 121 tcattttta accaataggc cgaaatcggt aaaatccctt ataaatcaaa agaatagacc
 181 gagatagggt tgagtgttgc tccaggttgg aacaagagtc cactattaaa gaacgtggac
 241 tccaaacgtca aaggcgaaa aaccgtctat cagggcgatg gcccactacg tgaaccatca
 301 cccaaatcaa gtttttgcg gtcgaggtgc cgtaaagctc taaatcgaa ccctaaaggg
 361 agcccccgat tttagagcttgc acggggaaag ccggcgaaacg tggcgagaaa ggaagggaaag
 421 aaagcgaaag gaggcgccg tagggcgctg gcaagtgttag cggtcacgct ggcgtaaacc
 481 accacaccccg cccgccttaa tgcgcgcgtc cagggcgctg ccattcgcca ttcaaggctgc
 541 gcaactgttgc ggaaggcgta tcggcgccgg ccttctcgat attacgcccag ctggcgaaag
 601 ggggatgtgc tgcaaggcgta ttaagtggg taacggccagg gttttccagg tcacgcgtt
 661 gtaaaacgcg ggcgcgttgc ttgttaatcgt actcactat ggcgaattt aaaaaacccc
 721 tcaagaccccg ttagaggcc ccaagggttgc atgctgtatc attctgcagg gtacccgggg
 781 atcctctaga gatccctcgat ctcgcgatc cattgtgggt gatttctacc aaggctagca
 841 tgggcagccg aatacagtga tccgtccgg ccctggactg ttgaacgagg tcggcgtaga
 901 cggtctgcg acacgcaaaac tggcgaaacg gttgggggtg cagcagccgg cgctttactg
 961 gcacttcagg aacaagcggg cgctgtcgat cgcactggcc gaagccatgc tggcgagaa
 1021 tcatacggtt cgggtccggag acggcgcgtc gactggcgat catttctgtat cgggaatccc
 1081 gcagccatgc tagcttgcgat aaaaattccac cacaatggat tcggaggat cttccatacc
 1141 taccagtttgc ggcgcgtcgc gtcgcggcccg cgactctctat gacgcgttaag cttactagca
 1201 taacccttgc gggcctctaa acgggtcttgc aggggttttt tgagtttctc gcccstatagt
 1261 gagtcgtatt acagcttgcgat tattctatag tgcacccatc atagcttggc gtaatcatgg
 1321 tcatacgcttgc ttccgtgtgc aatttgcgtt cgcgtccatc ttccacacaa catacgagcc
 1381 ggaagcataa agtgttgcgat ctgggggtgcg taatgagtgc gctaactcactc attaatttgcg
 1441 ttgcgtcact tgccgcgttgc cagtcgggg aacctgtcgat gccagctgcg ttaatgaaatc
 1501 ggcgaaacgcg cggggagagg cggttgcgttgc atggggcgat ctcgcgttca ctcgcgtact
 1561 gactcgctgc gtcggcgatc tccgtccggcg cgagcggtat cagtcgtactc aaaggcggt
 1621 atacgggttat ccacagaatc aggggataac gcaggaaaaga acatgtgagc aaaaggccag
 1681 caaaaggccca ggaaccgtaa aaggccgcg ttgcggcgat tttcgatag gctccggccc
 1741 cctgacgcg atcacaaaaaa tcgacgcgtca agtcagagg ggcgaaaccc gacaggacta
 1801 taaagatacc aggcgttcc ccctggaaage tccctcgatc gctctccgtt tccgacccctg
 1861 ccgcttacccg gatacctgtc cgccttctc ccttcggggaa gctggcgctt ttctcatagc
 1921 tcacgctgtatc ggtatctcgat tcgggtgtat gtcgttcgtt ccaagctggg ctgtgtgcac
 1981 gaaccccccgcg ttcaagccgcg ccgtcgccgc ttatccgtatc actatcgatc tgagtccaa
 2041 ccggtaagac acgacttatac ggcactggca gcagccactg gtaacaggat tagcaagagcg
 2101 aggtatgtatc gcggtgtatc aggttcttgc aagtgggtgc ctaactacgg ctacactaga
 2161 aggacagttat ttggatctgc cgcctcgatc aagccagtttgc ctttcggaaa aagagttgg
 2221 agcttctgtatc cccggaaacca aaccaccgcg ggtagcggtt gttttttgtt ttgcaagcag
 2281 cagattacgc gcagaaaaaaa aggtatcttgcgaaatccctt tgatcttttc tacgggtct
 2341 gacgctcgtatc ggaacaaaaa ctcacgtttaa gggattttttgcgatcgttgcgatcgttgc
 2401 atcttcacccatc agatccctttt aattttttttttaa tgaaggatcgttgcgatcgttgc
 2461 gagtaaactt ggtctgcacatc ttaccaatgc ttaatcgttgcgatcgttgcgatcgttgc
 2521 tgtctatttc gttcatccatc agttgcgttgc cttcccgatcgttgcgatcgttgcgatcgttgc
 2581 gagggcgatc catctggccccc cagtcgtcgcgatcgttgcgatcgttgcgatcgttgc
 2641 ccagatccatc cagcaataaa ccagcccgatcgttgcgatcgttgcgatcgttgc
 2701 actttatccgc ctcacatccgc gtcgttgcgatcgttgcgatcgttgcgatcgttgc
 2761 ccagatccatc gtttcggccatc cgttgcgttgcgatcgttgcgatcgttgcgatcgttgc
 2821 tcggtttggatc tggcttcattc cagtcgttgcgatcgttgcgatcgttgcgatcgttgc
 2881 cccatgttgcgatcgttgcgatcgttgcgatcgttgcgatcgttgcgatcgttgc
 2941 ttggccgcgcgatcgttgcgatcgttgcgatcgttgcgatcgttgcgatcgttgc
 3001 ccatccgtaa gatgttttgcgatcgttgcgatcgttgcgatcgttgcgatcgttgc
 3061 cgcgccccgcgatcgttgcgatcgttgcgatcgttgcgatcgttgcgatcgttgc
 3121 agcagaactt taaaatgtatcgttgcgatcgttgcgatcgttgcgatcgttgc
 3181 atcttacccgcgatcgttgcgatcgttgcgatcgttgcgatcgttgcgatcgttgc
 3241 gcatcttgcgatcgttgcgatcgttgcgatcgttgcgatcgttgcgatcgttgc
 3301 aaaaaggggaa taaggcgatcgttgcgatcgttgcgatcgttgcgatcgttgc
 3361 tattgaagatcgttgcgatcgttgcgatcgttgcgatcgttgcgatcgttgc
 3421 aaaaataaaac aaatagggttgcgatcgttgcgatcgttgcgatcgttgc
 3481 gaaaccatcgttgcgatcgttgcgatcgttgcgatcgttgcgatcgttgc
 3541 ctcgcgcgttgcgatcgttgcgatcgttgcgatcgttgcgatcgttgc
 3601 acagcttgcgatcgttgcgatcgttgcgatcgttgcgatcgttgc
 3661 gttggcggttgcgatcgttgcgatcgttgcgatcgttgcgatcgttgc

pGN49A

FIG. 14.

TGTAATACGA CTCACTATAG GGCGAATTCA AAAAACCCCT CAAGACCCGT
TTAGAGGCC CAAGGGGTTA TGCTAGTGAA TTCTGCAGCG GTACCCGGGG
ATCCTCTAGA GATCCCTCGA CCTCGAGATC CATTGTGCTG GAAAGGATCT
GGATCCGGCT TACTAAAAGC CAGATAACAG TATGCGTATT TGCGCGCTGA
TTTTTGCGGT ATAAGAATAT ATACTGATAT GTATACCCGA AGTATGTCAA
AAAGAGGTGT GCTATGAAGC AGCGTATTAC AGTGACAGTT GACAGCGACA
GCTATCAGTT GCTCAAGGCA TATATGATGT CAATATCTCC GGTCTGGTAA
GCACAACCAT GCAGAAATGAA GCCCCGTCGTC TGCGTGCCGA ACGCTGGAAA
GCGGAAAATC AGGAAGGGAT GGCTGAGGTC GCCCCGGTTA TTGAAATGAA
CGGCTCTTT GCTGACGAGA ACAGGGACTG GTGAAATGCA GTTTAAGGTT
TACACCTATA AAAGAGAGAG CCGTTATCGT CTGTTTGTGG ATGTACAGAG
TGATATTATT GACACGCCCG GGCGACGGAT GGTGATCCCC CTGGCCAGTG
CACGTCTCTT AAGCGATAAAA GTCTCCCGTG AACTTTACCC GGTGGTGCAT
ATCGGGGATG AAAGCTGGCG CATGATGACC ACCGATATGG CCAGTGTGCC
GGTCTCCGTT ATCGGGGAAG AAGTGGCTGA TCTCAGCCAC CGCGAAAATG
ACATCAAAAA CGCCATTAAC CTGATGTTCT GGGGAATATA AATGTCAGGC
TCCCTTATAC ACAGCCTTTTC CAGCACAATG GATCTCGAGG GATCTTCCAT
ACCTACCAGT TCTGCGCCTG CAGGTCGCGG CCGCGACTCT AGACCGCTAA
GCTTACTAGC ATAACCCCTT GGGGCCTCTA AACGGGTCTT GAGGGGTTTT
TTGAGCTTCT CGCCCTATAG TGAGTCGTAT TACAGCTTGA GTATTCTATA
GTGTCACCTA AATAGCTTGG CGTAATCATG GTCATAGCTG TTTCTGTGT
GAAATTGTTA TCCGCTCACA ATTCCACACA ACATACGAGC CGGAAGCATA
AAGTGTAAAG CCTGGGGTGC CTAATGAGTG AGCTAACTCA CATTAAATTGC
GTTGCGCTCA CTGCCCCTT TCCAGTCGGG AAACCTGTCG TGCCAGCTGC
ATTAATGAAT CGGCCAACGC GCGGGGAGAG GCGGTTTGCG TATTGGGCGC
TCTTCCGCTT CCTCGCTCAC TGACTCGCTG CGCTCGGTG 1TCCGCTGCG
GCGAGCGGT A TCAGCTCACT CAAAGGGGT AATACGGTTA TCCACAGAAT
CAGGGGATAA CGCAGGAAAG AACATGTGAG CAAAAGGCCA GCAAAAGGCC
AGGAACCGTA AAAAGGCCGC GTTGCTGGG TTTTCGATA GGCTCCGCC
CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC
CGACAGGACT ATAAAGATAAC CAGGCCTTTC CCCCTGGAAG CTCCCTCGTG
CGCTCTCCG TTCCGACCCCT GCGCCTTACG GGATACCTGT CCGCCTTCT
CCCTCGGGGA AGCGTGGCGC TTTCTCATAG CTCACGCTGT AGGTATCTCA
GTTCGGTGTA GGTGCTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC
GTTCAGCCCG ACCGCTGCGC CTTATCCGT AACTATCGTC TTGAGTCCAA
CCCGGTAAGA CACGACTTAT CGCCACTGGC ACCAGCCACT GGTAACAGGA
TTAGCAGAGC GAGGTATGTA GGCGGTGCTA CAGAGTCTT GAAAGTGGTGG
CCTAACTACG GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT
GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC
AAACCACCGC TGGTAGCGGT GGTTTTTTG TTTGCAAGCA GCAGATTACG
CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC
TGACGCTCAG TGGAAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT
TATCAAAAAG GATTTCAACC TAGATCCTTT TAAATTAAAA ATGAAGTTT

FIG. 14 (CONTINUED.)

AAATCAATCT AAAGTATATA TGAGTAAACT TGGCTTGACA GTTACCAATG
CTTAATCACT GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA
TAGTTGCCCTG ACTCCCCGTC GTGTAGATAA CTACGATACG GGAGGGCTTA
CCATCTGGCC CCAGTGCTGC AATGATACCG CGAGACCCAC GCTCACCGGC
TCCAGATTTA TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA
GTGGTCTCTGC AACTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG
GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT AGTTTGCAGCA ACGTTGTTGG
CATTGCTACA GGCATCGTGG TGTCACGCTC GTCGTTGGT ATGGCTTCAT
TCAGCTCCGG TTCCCAACGA TCAAGGCAGG TTACATGATC CCCCATGTTG
TGCAAAAAAG CGGTTAGCTC CTTCGGTCCT CCGATCGTTG TCAGAAGTAA
GTTGGCCGCA GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC
TTACTGTCAT GCCATCCGTA AGATGCTTT CTGTGACTGG TGAGTACTCA
ACCAAGTCAT TCTGAGAATA CCGCGCCCCG CGACCGAGTT GCTCTTGCCC
GGCGTCAATA CGGGATAATA GTGTATGACA TAGCAGAACT TTAAAAGTGC
TCATCATTGG AAAACGTTCT TCAGGGCGAA AACTCTCAAG GATCTTACCG
CTGTTGAGAT CCAGTTCGAT GTAACTTCACT CGTGCACCCA ACTGATCTTC
AGCATCTTTT ACTTTCACCA GCGTTCTGG GTGAGCAAAA ACAGGAAGGC
AAAATGCCGC AAAAAGGGGATAAGGGCGA CACGGAAATG TTGAATACTC
ATACTCTTCC TTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT
CATGAGCGGA TACATATTG AATGTATTAA GAAAAATAAA CAAATAGGGG
TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC CTGACGTCTA AGAAACCATT
ATTATCATGA CATTAAACCTA TAAAAATAGG CGTATCACGA GGCCCTTTCG
TCTCGCCGT TTCCGTGATG ACGGTGAAAA CCTCTGACAC ATGCAGCTCC
CGGAGACGGT CACAGCTTGT CTGTAAGCGG ATGCCGGGAG CAGACAAGCC
CGTCAGGGCG CGTCAGCGGG TGTGGCGGG TGTGGGGCT GGCTTAACTA
TGCGGCATCA GAGCAGATTG TACTGAGAGT GCACCATATG CGGTGTGAAA
TACCGCACAG ATGCGTAAGG AGAAAATACC GCATCAGGCG AAATTGTA
CGTTAATATT TTGTTAAAAT TCGCGTTAAA TATTGTTAA ATCAGCTCAT
TTTTAACCA ATAGGCCAA ATCGGCAAAA TCCCTTATAA ATCAAAAGAA
TAGACCGAGA TAGGGTGTGAG TGTGTTCCA GTTGGAAACA AGAGTCCACT
ATTAAAGAAC GTGGACTCCA ACGTCAAAGG GCGAAAAACC GTCTATCAGG
GCGATGGCCC ACTACGTGAA CCATCACCCA AATCAAGTTT TTTGCGGTCC
AGGTGCGTA AAGCTCTAAA TCGGAACCCCT AAAGGGAGCC CCCGATTTAG
AGCTTGACGG GGAAAGCCGG CGAACGTGGC GAGAAAGGAA GGGAAAGAAAG
CGAAAGGAGC GGGCGCTAGG GCGCTGGCAA GTGTAGCGGT CACGCTGCGC
GTAACCACCA CACCCGCCGC GCTTAATGCG CCGCTACAGG GCGCGTCCAT
TCGCCATTCA GGCTGCGCAA CTGTTGGAA GGGCGATCGG TGCAGGGCCTC
TTCGCTATTA CGCCAGCTGG CGAAAGGGGG ATGTGCTGCA AGGCGATTAA
GTTGGGTAAC GCCAGGGTTT TCCCAGTCAC GACGTTGTA AACGACGGCC
AGTGAAT

PGN594 FIG. 15.

GAGTGCACCA TATGCGGTGT GAAATACCGC ACAGATGCGT AAGGAGAAAA
TACCGCATCA GGCAGAATTG TAAACGTTAA TATTTTGTAA AAATTCGCGT
TAAATATTG TTAAATCAGC TCATTTTTA ACCAATAGGC CGAAATCGGC
AAAATCCCTT ATAAATCAA AGAATAGACC GAGATAGGGT TGAGTGTGTTG
TCCAGTTGG AACAAGAGTC CACTATTAAA GAACGTGGAC TCCAACGTCA
AAGGGCGAAA AACCCTCTAT CAGGGCGATG GCCCACTACG TGAACCCTCA
CCCAAATCAA GTTTTTGCG GTCGAGGTGC CGTAAAGCTC TAAATCGGAA
CCCTAAAGGG AGCCCCCGAT TTAGAGCTTG ACGGGGAAAG CCGGCGAACG
TGGCGAGAAA GGAAGGGAAG AAAGCGAAAG GAGCGGGCGC TAGGGCGCTG
GCAAGTGTAG CGGTACGCT GCGCGTAACC ACCACACCCG CCGCGCTTAA
TGCAGCCGCTA CAGGGCGCGT CCATTCGCCA TTCAGGCTGC GCAACTGTTG
GGAAGGGCGA TCGGTGCGGG CCTCTTCGCT ATTACGCCAG CTGGCGAAAG
GGGGATGTGC TGCAAGGCGA TTAAGITGGG TAACGCCAGG GTTTTCCCAG
TCACGACGTT GTAAAACGAC GGCAGTGAA TTGTAATACG ACTCACTATA
GGGCGAATTC GAGCTCGGT CCCGGGGATC CTCTAGAGAT CCCTCGACCT
CGAGATCCAT TGTGCTGGAA AGGATCTGGA TCCGGCTTAC TAAAGCCAG
ATAACAGTAT GCGTATTGTC GCGCTGATTT TTGCGGTATA AGAATATATA
CTGATATGTA TACCCGAAGT ATGTCAAAAA GAGGTGTGCT ATGAAGCAGC
GTATTACAGT GACAGTTGAC AGCGACAGCT ATCAGTTGCT CAAGGCATAT
ATGATGTCAA TATCTCCGGT CTGGTAAGCA CAACCATGCA GAATGAAGCC
CGTCGTCTGC GTGCCGAACG CTGGAAAGCG GAAAATCAGG AAGGGATGGC
TGAGGTCGCC CGGTTTATTG AAATGAACGG CTCTTTTGCT GACGAGAAC
GGGACTGGTG AAATGCAGTT TAAGGTTTAC ACCTATAAAA GAGAGAGCCG
TTATCGTCTG TTTGTGGATG TACAGAGTGA TATTATTGAC ACGCCCGGGC
GACGGATGGT GATCCCCCTG GCCAGTGCAC GTCTCTTAAG CGATAAAAGTC
TCCCGTGAAC TTTACCCGGT GGTGCATATC GGGGATGAAA GCTGGCGCAT
GATGACCACC GATATGCCA GTGTGCCGGT CTCCGTTATC GGGGAAGAAG
TGGCTGATCT CAGCCACCGC GAAAATGACA TCAAAAACGC CATTAACTCG
ATGTTCTGGG GAATATAAAT GTCAGGCTCC CTTATACACA GCCTTCCAG
ACAATGGAT CTCGAGGGAT CTTCCATACC TACCAGTTCT GCGCCTGCAG
GTGCGGGCC CGACTCTCTA GAGTCGAAAG CTTCTCGCC TATAGTGACT
CGTATTACAG CTTGAGTATT CTATAGTGTG ACCTAAATAG CTTGGCGTAA
TCATGGTCAT AGCTGTTTCC TGTGTGAAAT TGTATCCGC TCACAATTCC
ACACAACATA CGAGCCGGAA GCATAAAAGTG TAAAGCCTGG GGTGCCTAAT
GAGTGAGCTA ACTCACATTA ATTGCGTTGC GCTCACTGCC CGCTTCCAG
TCGGGAAACC TGTCGTGCCA GCTGCAITAA TGAATCGGCC AACGCGCGGG
GAGAGGCGGT TTGCGTATTG GGCCTCTC CGCTTCCCTCG CTCACTGACT
CGCTGCCGCTC GGTGCTTCGG CTGCCGGAG CGGTATCAGC TCACTCAAAG
GCCGTAATAC GGTTATCCAC AGAATCAGGG GATAACGCGAG GAAAGAACAT
GTGAGCAAA GGCCAGCAAA AGGCCAGGAA CGCTAAAAG GCGCGGTG
TGGCGTTTTT CGATAGGCTC CGCCCCCTG ACGAGCATA CAAAAATCGA
CGCTCAAGTC AGAGGTGGCG AAACCCGACA GGACTATAAA GATACCAGGC
GTTTCCCCCT GGAAGCTCCC TCGTGCCTC TCCGTGTTCCG ACCCTGCCGC
TTACCGGATA CCTGTCCGCC TTTCTCCCTT CGGGAAGCGT GGCGCTTTCT

FIG. 15 (CONTINUED)

CATAGCTCAC GCTGTAGGTA TCTCAGITCG GTGTAGGTCG TTCGCTCCAA
GCTGGGCTGT GTGCACGAAC CCCCCGTTCA GCCCGACCGC TGCGCCTTAT
CCGGTAACCA TCGTCTTGAG TCCAACCCGG TAAGACACGA CTTATCGCCA
CTGGCAGCAG CCACTGGTAA CAGGATTAGC AGAGCGAGGT ATGTAGGCAG
TGCTACAGAG TTCTTGAAAGT GGTGGCCTAA CTACGGCTAC ACTAGAAGGA
CAGTATTGAG TATCTGCGCT CTGCTGAAGC CAGTACCTT CGGAAAAAGA
GTTGGTAGCT CTTGATCCGG CAAACAAACC ACCGCTGGTA GCGGTGGTTT
TTTTGTTGCA AAGCAGCAG TTACGCGAG AAAAAAAGGA TCTCAAGAAG
ATCCCTTGAT CTTTCTACG GGGTCTGACG CTCAGTGGAA CGAAAAACTCA
CGTTAAGGAA TTTTGGTCAT GAGATTATCA AAAAGGATCT TCACCTAGAT
CCTTTAAAT TAAAAATGAA GTTTTAAATC AATCTAAAGT ATATATGAGT
AAACTGGTC TGACAGTTAC CAATGCTTAA TCAGTGAGGC ACCTATCTCA
GCGATCTGTC TATTCGTTTC ATCCATAGTT GCCTGACTCC CCGTCGTGTA
GATAACTACG ATACGGGAGG GCTTACCATC TGGCCCCAGT GCTGCAATGA
TACCGCGAGA CCCACGCTCA CCGGCTCCAG ATTTATCAGC AATAAACCAG
CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT CCTGCAACTT TATCCGCCCTC
CATCCAGTCT ATTAATTGTT GCGGGAAAGC TAGAGTAAGT AGTTGCCAG
TTAATAGTTT GCGCAACGTT GTTGGCATTG CTACAGGCAT CGTGGTGTCA
CGCTCGTCGT TTGGTATGGC TTCATTCAAGC TCCGGTTCCC AACGATCAAG
GCGAGTTACA TGATCCCCCA TGTTGTGCAA AAAAGCGGTT AGCTCCTTCG
GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG CCGCAGTGGT ATCACTCATG
GTTATGGCAG CACTGCATAA TTCTCTTACT GTCATGCCAT CCGTAAGATG
CTTTCTGTG ACTGGTGTAGT ACTCAACCAA GTCATTCTGA GAATACCGCG
CCGGCCGACC GAGTTGCTCT TGCCCGCGT CAATACGGGA TAATAGTGTAA
TGACATAGCA GAACCTTAAA AGTGCTCATC ATTGGAAAAGC GTTCTCGGG
GCGAAAACTC TCAAGGATCT TACCGCTGTT GAGATCCAGT TCGATGTAAC
CCACTCGTGC ACCCAACTGA TCTTCAGCAT CTTTACTTT CACCAGCGTT
TCTGGGTGAG CAAAAACAGG AAGGCAAAT GCGCAAAAA AGGGATAAG
GGCGACACGG AAATGTTGAA TACTCATACT CTTCTTTT CAATATTATT
GAAGCATTTA TCAGGGTTAT TGTCTCATGA GCGGATAACAT ATTTGAATGT
ATTTAGAAAA ATAAACAAAT AGGGGTTCCG CGCACATTTC CCCGAAAAGT
GCCACCTGAC GTCTAAGAAA CCATTATTAT CATGACATTA ACCTATAAAA
ATAGGGCTAT CACGAGGCCC TTTCGTCCTCG CGCGTTTCGG TGATGACGGT
GAAAACCTCT GACACATGCA GCTCCCGAG ACGGTCACAG CTTGTCTGTAA
AGCGGATGCC GGGAGCAGAC AAGCCCGTCA GGGCGCGTCA GCGGGTGTGTT
GCGGGTGTGCG GGGCTGGCTT AACTATGCGG CATCAGAGCA GATTGTACTG

A

FIG. 16.

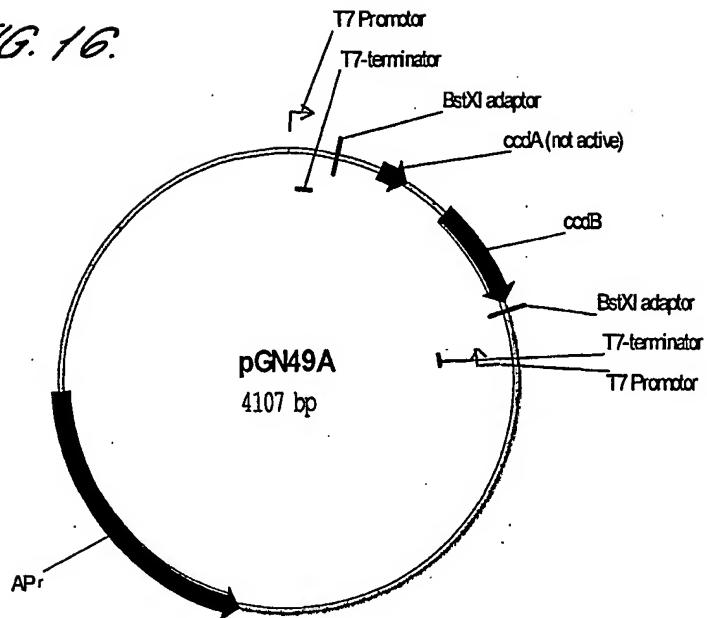
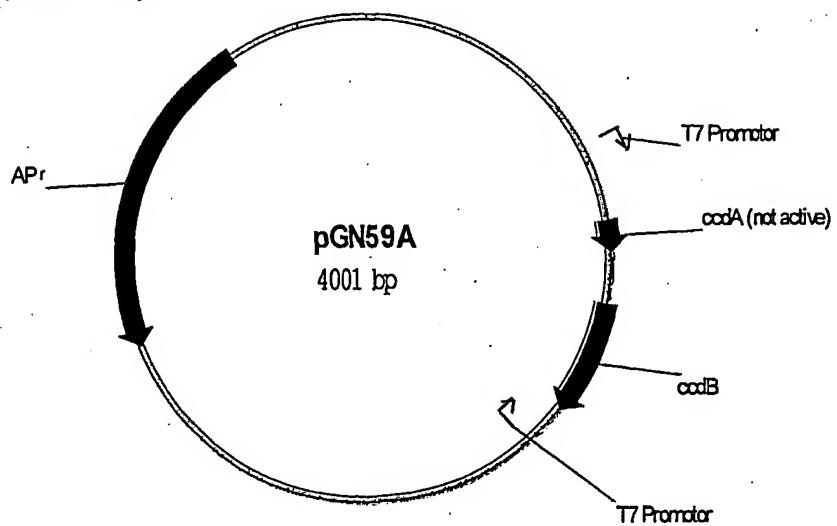


FIG. 17.



SEQUENCE LISTING

<110> DEVGEM NV

<120> VECTOR CONSTRUCTS

<130> SCB/55178/001

<140>

<141>

<160> 21

<170> PatentIn Ver. 2.0

<210> 1

<211> 160

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Fragment of
pGN1 containing opposable T7 promoters

<400> 1

ttgttaatacg actcaactata gggcgaattc gagctcggtt cccggggatc ctcttagagtc 60
gaaagcttct cgccctatag ttagtcgtat tacagcttga gtattctata gtgtcaccta 120
aatagcttgg cgtaatcatcg gtcatacgctg tttcctgtgt 160

<210> 2

<211> 49

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: DNA sequence
containing a T7 terminator

<400> 2

actagcataa ccccttgggg cctctaaacg ggtcttgagg ggtttttg

49

<210> 3

<211> 70

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide oGN27

<400> 3

aattcaaaaa acccctcaag acccgtttag agggcccaag gggttatgct agtgaattct 60
gcagcggtag 70

<210> 4

<211> 62

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:

Oligonucleotide oGN28

<400> 4

cgctgcagaa ttcactagca taaccccttg gggcctctaa acgggtcttg aggggtttt 60
tg 62

<210> 5

<211> 65

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:

Oligonucleotide oGN29

<400> 5

ctagacgcgt aagcttacta gcataacccc ttggggcctc taaacgggtc ttgaggggtt 60
ttttg 65

<210> 6

<211> 65

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:

Oligonucleotide oGN30

<400> 6

agctaaaaaa acccctcaag acccgtagg aggcccaag gggttatgtc agtaagctt 60
cgcgt 65

<210> 7

<211> 230

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Fragment of
plasmid pGN9 containing opposable T7 promoters and
T7 transcription terminators

<400> 7

ttgttaatacg actcaactata gggcgaattc aaaaaacccc tcaagacccg ttttagaggcc 60
ccaagggtt atgctagtga attctgcagg gtacccgggg atccctctaga cgcgtaaatc 120
tactagcata acccccttggg gcctctaaac gggtcttgag gggtttttg agcttctcgc 180
cctatagtga gtcgtattac agcttgagta ttctatagtg tcacctaaat 230

<210> 8

<211> 3323

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGN9

<400> 8

gagtgcacca tatgcgggtgt gaaataccgc acagatgcgt aaggagaaaa taccgcata 60
ggcggaaatg taaacgttaa tattttgtta aaattcgcgt taaatatttg ttaaatcagc 120
tcattttta accaataggc cgaaatcgcc aaaatccctt ataaatcaaa agaatacgacc 180
gagatagggt tgagtgtgt tccagtttg aacaagatgc cactattaaa gaacgtggac 240

tccaacgtca aaggcgaaa aaccgtctat cagggcgatg gcccactacg tgaaccatca 300
 cccaaatcaa gtttttgcg gtcgagggtgc cgtaaagctc taaatcgaa ccctaaagg 360
 agcccccgtat ttagagcttgc acggggaaag cccgcgaacg tggcgagaaa ggaaggaaag 420
 aaagcgaaag gagcggcgctc tagggcgctg gcaagtgtacg cggtcacgct gcgcgttaacc 480
 accacacccg ccgcgtttaa tgcgcgcata cagggcgctg ccattcgcca ttcaggctgc 540
 gcaactgttgc ggaaggcgatc tcgggtcgccc cctttcgctt attacgcacg ctggcgaaag 600
 gggatgtgc tgcaaggcgatc ttaagttggg taacgcagg gtttcccgatc tcacgacgtt 660
 gtaaaacgac ggcgcgttgc ttgtataatcg actcaactata gggcgaattc aaaaaacccc 720
 tcaagacccg ttttagggcc ccaagggttgc atgctgtacg attctgcagg gtacccgggg 780
 atcctctaga cgcgtaaatc tactagcata accccttggg gcctctaaac gggcttttag 840
 gggtttttgc agcttctcgatc cctataatcgatc gtcgttattac agcttgcgtt 900
 tcacctaataatc agcttgcgttgc aatcatggtc atagctgtttt cctgtgtgaa attgttatcc 960
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<211> 3774

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGN29

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<211> 5148
<212> DNA
<213> Artificial Sequence

<220>
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<223> Description of Artificial Sequence: Plasmid
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<211> 4001

<212> DNA

<213> Artificial Sequence

<220>

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<211> 34

<212> DNA

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<223> Description of Artificial Sequence:
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<400> 15

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<210> 16

<211> 40

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:
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<210> 17

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<212> DNA
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<210> 19
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<223> Description of Artificial Sequence:
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<223> Description of Artificial Sequence: PCR fragment
generated by primers oGN103 and oGN104 on pCDM8

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gtaccagcac aatgg 375

<210> 21
<211> 670
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR fragment

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 01/01068

A. CLASSIFICATION OF SUBJECT MATTER		
IPC 7 C12N15/10 C12N15/63 C12N15/70 C12N1/21		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC 7 C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the International search (name of data base and, where practical, search terms used)		
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, MEDLINE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FR 2 782 325 A (PROTEUS) 18 February 2000 (2000-02-18) page 7, line 20 -page 8, line 8 page 11, line 11 - line 36 page 23, line 31 -page 24, line 9 ---	1-24, 26, 27
A	WO 00 01846 A (DEVGEM N.V.) 13 January 2000 (2000-01-13) cited in the application page 8, line 9 -page 10, line 22 page 15, line 9 - line 33 page 21, line 21 -page 22, line 29 ---	1-28 -/-
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.		<input checked="" type="checkbox"/> Patent family members are listed in annex.
<p>* Special categories of cited documents :</p> <p>'A' document defining the general state of the art which is not considered to be of particular relevance</p> <p>'E' earlier document but published on or after the international filing date</p> <p>'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>'O' document referring to an oral disclosure, use, exhibition or other means</p> <p>'P' document published prior to the international filing date but later than the priority date claimed</p> <p>'T' later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>'&' document member of the same patent family</p>		
Date of the actual completion of the international search	Date of mailing of the international search report	
20 September 2001	27/09/2001	
Name and mailing address of the ISA	Authorized officer	
European Patent Office, P.B. 5818 Palentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 01/01068

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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